EXTENSION OF CELL VIABILITY IN RESTED POST-MORTEM TELEOST WHITE MUSCLE

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CF	Condition factor
CPK	Creatine phosphokinase
FAD	Flavin adenine dinucleotide
GP	Glycogen phosphorylase
HSI	Hepatosomatic index
IMP	Inosine monophosphate
$\dot{M}O_2$	Standard oxygen consumption
NAD^+	Nicotinamide-adenine dinucleotide
PCr	Phosphocreatine
PFK	Phosphofructokinase
P_i	Inorganic phosphate
PK	Pyruvate kinase
PM	Post-mortem
RCR	Respiratory Control Ratio
WM	White muscle

ABSTRACT

The objective of this thesis was to investigate how cell viability can be maintained in rested ischemic teleost white muscle (WM) during post-mortem (PM) storage. For cells to remain viable they require energy in the form of ATP for cellular processes. Exercise prior to anaesthesia severely depleted the ATP generating potential of the WM (estimated by WM pH) from yellow-eye mullet (Aldrichetta forsteri) prior to PM storage, reducing the pre-rigor period. Fish anaesthetised in a rested state had a high ATP generating potential (intact ATP, PCr and glycogen WM stores) with ATP being able to be supplied to cells during PM storage, extending the pre-rigor period. To further extend cell viability during PM storage either the rate of ATP utilisation needed to be curbed or more ATP needed to be generated in the WM. As aerobic generation of ATP is more efficient than anaerobic, an attempt was made to deliver oxygen to the ischemic WM during PM storage. WM from rested mullet was stored under hyperbaric, hyperoxic conditions (620 ± 10 kPa with humidified oxygen flow of 50 mL/min ± 1.25%) and the rate of acidification was slowed by two-thirds compared with storage under normobaric, hyperoxic conditions (humidified oxygen flow of 50 mL/min ± 1.25%). Onset of ATP depletion and lactate accumulation was delayed for ~27 h suggesting a period of aerobic ATP generation. In chinook salmon (Oncorhynchus tshawytscha) the delay was ~12 h and in snapper (Pagrus auratus) ~37 h. Physical condition and functional differences between species played a significant role in determining the benefit from the storage treatment.

As aerobic ATP generation did not continue indefinitely in the hyperbaric, hyperoxic WM, the investigation turned to the site of oxidative phosphorylation (the mitochondria) in order to determine what inhibits their function during PM storage. Varying the incubation medium pH and CO_2 concentration (over a physiological range) did not inhibit mitochondria respiration in vitro. Only very high levels of CO_2 in combination with low pH may inhibit mitochondria. Extracting mitochondria from WM during PM storage showed that mitochondria from hyperbaric, hyperoxic WM may become damaged due to the high levels of oxygen, consequently leading to an inhibition of oxidative phosphorylation.

Cold acclimation of fish often results in compensatory biochemical modifications to maintain aerobic flux. It was hypothesised that fish acclimated to cold temperatures could generate ATP aerobically for longer when stored under hyperbaric, hyperoxic conditions. However, the PM period of aerobic ATP generation was greatest in yellow-eye mullet acclimated to summer temperatures. Winter mullet may rely solely on carbohydrate to be used for both aerobic and anaerobic ATP generation, whereas in summer both lipid and carbohydrate, may be available, i.e. summer mullet have more available substrate.

CHAPTER 1

General introduction and literature review

1.1 General Introduction

All animals require a constant supply of energy to sustain life. Energy is obtained mostly through the oxidation of foodstuffs or through body stores when feed is limited. The common energy currency of living organisms is adenosine triphosphate (ATP) (Schmidt-Nielsen 1993). Hydrolysis of the "energy rich" phosphate bond in ATP provides the immediate energy source for energy-requiring processes and reactions. ATP can be generated using a variety of reactions mainly involving metabolism with oxygen, but also via anaerobic processes.

Glucose is the major carbohydrate fuel for most cells and is a common metabolic substrate for energy generation. In the cell, glucose metabolism begins with glycolysis: the conversion of glucose (six-carbon sugar) to pyruvate (three carbon sugar) via a series of biochemical reactions, through the Embden-Meyerhof pathway. Sugars other than glucose can also be utilised by being converted to intermediates of glycolysis (Mathews & van Holde 1990). Glycolysis proceeds under anaerobic conditions (a major focus of the thesis) but for the complete combustion of glucose to CO_2 and water pyruvate is oxidised to acetyl-CoA, with the final oxidation of the acetyl group carbons in the citric acid cycle (TCA cycle). The energy release occurs in the mitochondria and is in the form of exergonic dehydrogenation reactions that generate reduced electron carriers; these carriers are next reoxidised in the mitochondrial respiratory chain (electron transport chain; Mathews & van Holde, 1990). This is known as oxidative phosphorylation. Overall, energy production from glucose can be summarized in Fig. 1.1.

The under lying theme that runs through this thesis is how cell viability can be maintained in rested ischemic fish white muscle (WM) during post-mortem (PM) storage and where the energy required (in the form of ATP) for viability is coming from. Is it entirely derived anaerobically or is some of the ATP generated through aerobic means? Is ATP being consumed unnecessarily through inefficiencies or futile cycling and if so, how can it be conserved?

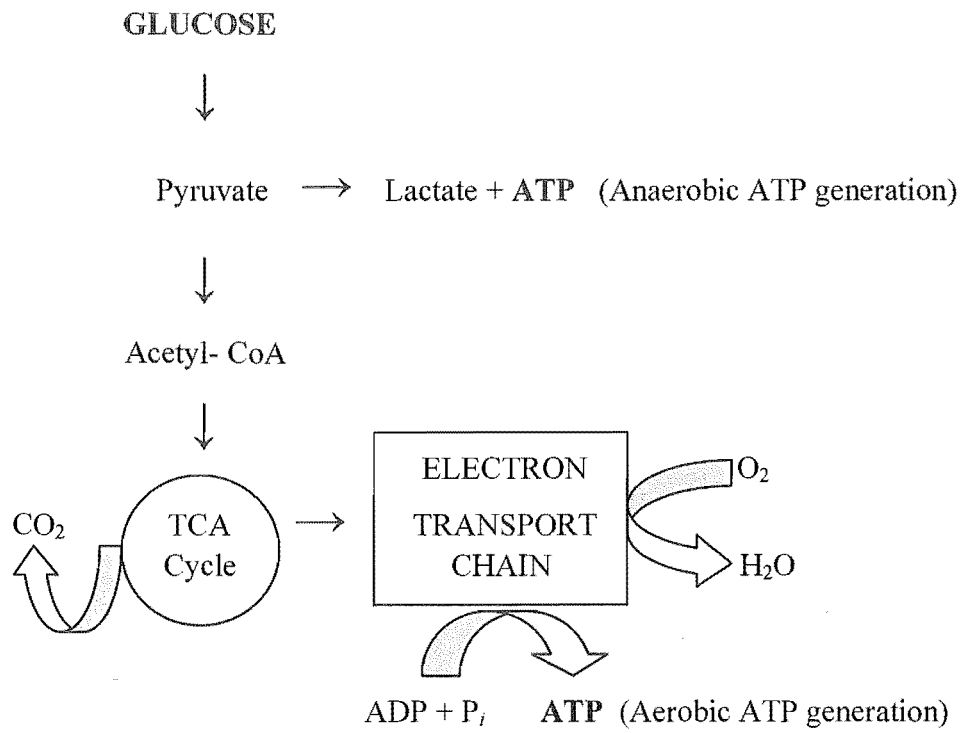

Figure 1.1: *Energy production from glucose.*

While a fish is alive the majority of the energy is generated via oxidative phosphorylation, as it is in most animals. When energy demand is high, e.g. burst exercise, large amounts of ATP are required, at a rate far greater than can be supplied by oxidative phosphorylation. To overcome this, pyruvate is reduced to lactate by lactate dehydrogenase, yielding ATP by anaerobic means.

When a fish is killed the tissue still remains "alive" for a period. The cells still try to maintain homeostasis with most processes requiring energy in the form of ATP. As ion imbalances occur the cell membrane becomes more permeable with even more energy required to keep the cell viable. Eventually the cells cease functioning due to the depletion of ATP and the loss of the cells ability to generate ATP to maintain homeostasis.

Once cell viability is lost, PM changes to the structure of the WM begin and continue for some time. The physical changes have traditionally been separated out into stages: rigor mortis (stiffening of the WM), dissolution of rigor mortis (WM softens again), autolysis and bacterial spoilage (Sigholt et al. 1997). The earliest changes to fish WM during storage that can be detected by the senses are those concerned with texture and appearance (Huss 1995). The most dramatic of these changes is the onset of

rigor mortis whereby the fish stiffens due to contraction of the musculature that cannot be relaxed. Actin and myosin filaments irreversibly bind together as there is no ATP to provide the energy needed for detachment (Schmidt-Nielsen 1993). The rate of onset of rigor mortis can be affected by temperature, handling, size and physical condition of the fish. Dissolution of rigor mortis results in a softening of the WM, however, the elastic properties the WM had prior to the development of rigor mortis are lost. During the resolution of rigor in the WM autolysis ("self-digestion") begins. There are two main types of fish spoilage: bacterial and enzymatic. Enzymatic spoilage occurs much earlier in PM fish WM than bacterial spoilage and is unrelated to microbially mediated processes (Huss 1995, Pedraja 1970). Due to these two processes adversely affecting the shelf life of the WM there has been extensive research into reducing the rate and onset of enzymatic autolysis (and concomitantly bacterial spoilage).

The same PM changes also occur in the red muscle of mammals. The major difference is that enzymatic autolysis is encouraged in mammalian muscle for tenderisation purposes. Firm texture and elasticity are two of the most desirable properties to be maintained in fish WM. Therefore inhibition or minimisation of these enzymatic processes in fish WM is paramount.

Once the onset of rigor mortis occurs there is little that can be done to prevent the continuation of physical changes and spoilage of the WM apart from reducing the rate of the changes. Rigor mortis only develops in the WM due to the decline in high-energy phosphate stores, i.e. ATP, during the initial period of PM storage. Therefore, extension of the "pre-rigor" state where energy reserves are conserved for as long as possible, (essentially preventing the onset of rigor mortis), is highly desirable. As this is the focus of the thesis there is a review of the basic structure, function and physiology of fish muscle as well as factors affecting the pre-rigor state of the WM.

1.2 Structure and function of red and white muscle

As in mammals, the muscle tissue of fish is composed of striated muscle. The muscle cell (myoblast) interior consists of the protein filaments (myofibrils, up to 1000, Alberts et al. 1994), which are surrounded by the sarcoplasma. The sarcoplasma contains the nuclei, mitochondria, the internal membrane systems of the sarcoplasmic reticulum and the T system, and a fuel store in the form of glycogen granules (Aidley, 1991). The

plasma membrane and a sheath of connective tissue, collectively called the sarcolemma, surround the cell. The myofibrils contain the contractile proteins, actin and myosin. These proteins or filaments are arranged in a characteristic alternating system making the muscle appear striated (Schmidt-Nielsen 1993). For the muscle to contract a nervous impulse sets off a release of Ca^{2+} from the sarcoplasmic reticulum that surrounds the contractile filaments and myofibrils. The contractile proteins myosin and actin in the myofibrils are then allowed to interact forming the basis of a contraction. The myosin will not detach from the actin strand until it is combined with a new ATP molecule (a more detailed description of the muscle contraction process has been made by Schmidt-Nielsen 1993). In the case of rigor mortis where the ATP supply has been depleted, the myosin cannot detach from the actin and thus, the muscle stays contracted.

In higher vertebrates the locomotory muscle contains a mixture of both slow twitch (red) and fast twitch (white) muscle fibres resulting in technical difficulty if research is to be carried out on only one fibre type. The discrete separation of muscle fibre types in fish muscle into homogeneous groupings has allowed researchers to take advantage of this structure for studying various aspects of muscle metabolism (Johnston 1981). The characterisation of the different fish muscle fibre types has traditionally been based on colour, with three groups of fibres being identified: red, white and an intermediary pink. The body mass of a typical salmonid is made up of 60% WM and has only 7% red and pink muscle (Moyes and West 1995). For the remainder of the study I will only be discussing the red and white fibres.

Red muscle (RM) fibres form a thin superficial layer, thickening to a V-shaped wedge at the major horizontal septum (Johnston 2001). RM has fibres that are very small in diameter (high surface to volume ratio) and have a good supply of capillaries (capillary: fibre ratio of 1.9-2.5) allowing efficient gas exchange. In WM the fibre diameter is larger (low surface to volume ratio) and have a capillary: fibre ratio of just 0.2-0.9. RM is characterized by its high mitochondrial content (25-35% of the fractional fibre volume) compared with <2% in WM (Johnston et al. 1975; Moyes and West 1995). The size of mitochondria in the RM are also larger than in WM. Moyes et al. (1989) found that carp RM had a 14-fold higher activity of citrate synthase than WM reflecting the higher mitochondrial density in this tissue. RM also has high concentrations of myoglobin (giving it a red colour), cytochromes and lipids (Johnston et al. 1975), with WM having lower myoglobin and having low levels of lipids, e.g.

Atlantic salmon WM has a lipid content of 2% whereas RM has about 15% (Navarro & Gitiérrez 1995). Moyes et al. (1989) showed that the most profound difference in the oxidative properties of WM and RM was in their ability to use fatty acids. RM was able to use pyruvate and fatty acyl carnitines equally well but in the WM the rate of pyruvate oxidation was 1.5–threefold greater than that of fatty acyl carnitine oxidation. Carnitine palmitoyl transferase activity in WM mitochondria is low and together with low lipid content suggests that lipid is a poor substrate for the WM. Overall, the RM has a much higher aerobic capacity than the WM. Associated with the high glycolytic capacity of WM fibres they have a myofibrillar ATPase (breaks down ATP to ADP) activity three times that of red fibres (Johnston 1975). ATPase activity parallels the unloaded speed of shortening of the muscle fibres. Thus WM fibres can contract much faster than red, hence the terminology of fast (white) and slow (red) muscle fibres.

1.3 Energy production in muscle

The energy "currency" in the muscle cell is ATP. The main ATP demand pathways in the cell and organelle membranes include the maintenance of ion gradients via ATP-dependent ion pumps (Na^+/K^+ pump, Ca^{2+} pump), protein synthesis and protein degradation (Heffron & Hegarty, 1974; Churchill et al. 1995; Hochachka 1997). These processes account for the majority of the ATP generated from O_2 consumption.

1.3.1 High energy phosphagens

When endogenous fuels are considered as those substances that contribute to cellular energy metabolism (i.e. supply ATP), the ultimate forms of energy are phosphorylated adenylates (especially ATP itself) and creatine phosphate (PCr) (van den Thillart & van Raaij 1995). During highest intensity muscle work the following reaction occurs:

$$\text{Phosphocreatine} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

Although the rate of ATP generation from these sources is rapid they can only provide enough energy for a few seconds of burst exercise due to the low levels of ATP and PCr stored in the WM (~10 μmol/g and 20–30 μmol/g, respectively; van den Thillart & van Raaij 1995).

1.3.2 Lipid

During sustained exercise, where fatigue is minimal, the RM is primarily used due to its high aerobic capacity (see above) with lipid being the major energy fuel. It has been estimated that during sub-maximal exercise, coho salmon would derive 45% of its energy from lipid catabolism whereas the value was decreased to about 15% during exhaustive exercise (van den Thillart & van Raaij 1995). In rainbow trout lipid is also the dominant substrate when the fish is at rest with its percentage contribution declining as swimming speed increased (Kieffer et al. 1998). Interestingly, at 5 °C carbohydrate was the preferred fuel for energy generation.

Lipids are stored in the muscle primarily as triglycerides with the RM also displaying substantial triglyceride lipase activity allowing the lipid in the muscle to be used for energy metabolism in situ as free fatty acids. Lipid breakdown generates acetyl-CoA primarily through β-oxidation of fatty acids.

1.3.3 Glycolysis

During burst exercise where the demand for ATP is at a peak the RM cannot sustain supply at a high enough flux to satisfy the demand and cannot generate sufficient force. As the WM makes up the bulk of the musculature it must be recruited for maximal thrust. WM has limited endogenous stores of ATP and PCr that are depleted rapidly (in a few seconds) during burst exercise (as described above). For a more extended period of energy supply during exercise carbohydrate in the form of glycogen is the preferred substrate. By its very structure (large fibre diameter, low numbers of mitochondria that are small in size, poor capillary supply, no myoglobin) WM is limited to using anaerobic metabolism to supply itself with ATP. Glycogen is stored in both red and WM cells and can be readily mobilized to pyruvate as needed.

Although the WM is specialised for burst type exercise it does not typically store more glycogen than the red fibres (van den Thillart & van Raaij 1995). Pande & Blanchaer (1971) found that in rabbit skeletal muscle the mitochondria oxidise pyruvate at a faster rate, giving a higher energy yield (ADP: oxygen ratio) than when oxidising fatty acids. Thus, carbohydrate oxidation has a greater potential for meeting severe energy demands. In situations of oxygen limitation, i.e. burst exercise, this is

particularly valuable. Because the supply of oxygen is limited, oxidation of carbohydrate would provide more energy than that of fat.

To breakdown glycogen (glycogenolysis) to allow it to be a substrate for glycolysis several steps are involved:

Glycogen (six-carbon sugar)

↓ *glycogen phosphorylase (GP)*

Glucose-1, 6-bisphosphate

↓ *phosphoglucomutase*

Glucose-6-phosphate (three carbon sugar phosphate)

Glycolysis has an initial energy investment phase (two ATPs required) to split the six-carbon substrate into two three-carbon sugar phosphates, followed by an energy generation phase with a net yield of 2 moles of ATP, 2 moles of NADH (reducing equivalent) and 2 moles of pyruvate for every mole of glucose metabolised:

$$\text{Glucose} \longrightarrow \text{Pyruvate}$$

$$2\text{ADP} \longrightarrow 2\text{ATP}$$

$$2\text{NAD}^+ \longrightarrow 2\text{NADH}$$

For a full summary of glycolysis see Fig. 1.2.

For glycolysis to continue operating anaerobically the NADH must be reoxidised to NAD^+ to maintain a steady state. The most straightforward route is by using NADH to reduce pyruvate to lactate, via the enzyme lactate dehydrogenase.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

Glycolysis can proceed under anaerobic conditions (a major focus of the thesis) but for complete oxidation of carbohydrates considerable oxygen consumption is required.

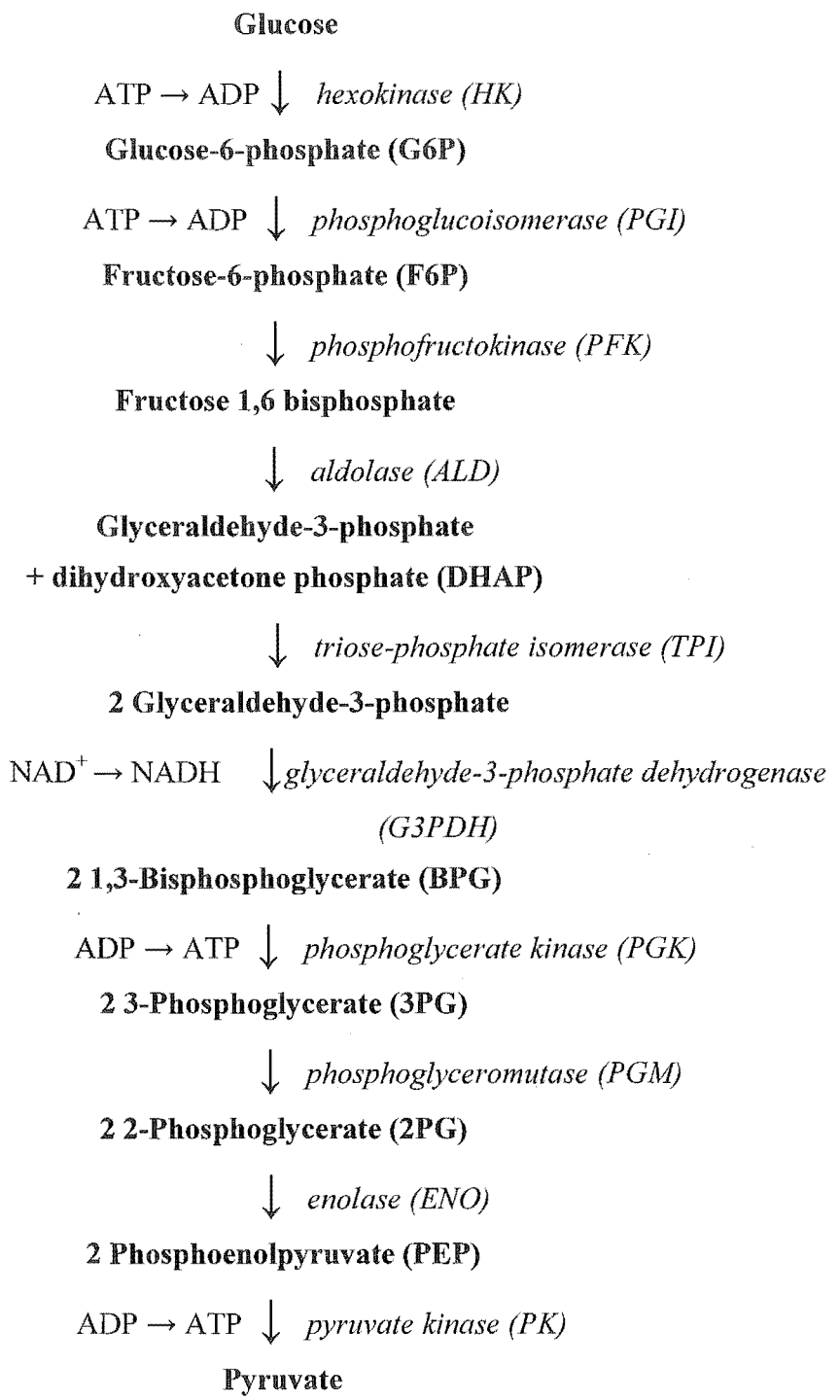

Fig 1.2: *Summary of glycolysis with enzymes in italics (from Mathews & van Holde 1990).*

For the complete combustion of glucose to CO_2 and water pyruvate is oxidised to acetyl-CoA, with the final oxidation of the acetyl group carbons in the citric acid cycle. One turn of the TCA cycle yields:

$$\text{Acetyl-CoA} + 3NAD^+ + FAD + ADP + P_i + 3H_2O \longrightarrow$$

$$3NADH + FADH_2 + \text{CoA-SH} + ATP + 3CO_2$$

(Mathews & van Holde 1990)

The reduced electron carriers (NADH, $FADH_2$) then become reoxidised in the electron transport chain, with concomitant synthesis of ATP. The phosphorylation of ADP to ATP is the key to the electron transport chain, and can be defined by the equation:

$$ADP^{3-} + P_i^{2-} + H^+ \rightarrow ATP^{4-} + H_2O$$

The electron transfer chain in oxidative phosphorylation is in reality a series of enzymes that are, by virtue of their molecular constitution, able to accept electrons. The complete system is quite complex, but the electron transfer chain has two main functions:

1) To pump H^+ ions across the inner mitochondrial membrane. This leads to a H^+ gradient which acts as the driving force for the conversion (phosphorylation) of ADP to ATP.

2) The completion of the oxidation of glucose through the transfer of electrons to molecular oxygen, the most powerful electron acceptor.

To maintain the oxidative metabolism of glucose there needs to be a constant supply of oxygen. Certain metabolic intermediates, e.g. lactate and pyruvate, can substitute for glucose as alternative substrates for metabolism but there is no such substitute for oxygen.

1.3.4 Control of glycolysis

Anaerobic glycolysis is regulated at various points in the glycolytic pathway including the enzymes glycogen phosphorylase (GP), phosphofructokinase (PFK) and pyruvate kinase (PK). In mammalian muscle the control of glycogen breakdown is hormonal.

The catecholamine adrenaline binds to adenylate cyclase on the cell membrane, initiating a cascade of reactions that mediates the activation or inhibition of phosphorylase kinase by cAMP dependent protein kinases or specific phosphatases (for a good description see Mathews & van Holde 1990). During adrenaline stimulation, the major part of glycogen phosphorylase is phosphorylated from the *b* form to the *a* (active) form in fast-twitch muscle (Jensen et al. 1999). However, Johnston (1981) discussed findings that dogfish GP depended only on Ca^{2+} for its activity (nerve firing depolarises the muscle cell membrane leading to Ca^{2+} release from the sarcoplasmic reticulum and contraction of the muscle, along with activation of GP to stimulate glycolysis for ATP supply). Johnston (1981) noted that due to the WM having relatively poor circulation during burst exercise it is not unexpected that the hormonal mechanism for glycogen breakdown would be lacking in the WM. In mammalian (rat) fast-twitch WM incubated with adrenaline, Jensen et al. (1999) found that 80% of GP was in the *a* form, i.e. there was adrenergic activation of glycogenolysis. Similarly, in rainbow trout WM, Mehrani (1998), found that exhaustive exercise resulted in a 40% increase in glycogen phosphorylase kinase (phosphorylates GP to the active form) and a 70% increase in cellular cAMP levels. This also suggests a hormonal activation of glycogenolysis in the WM. Johnston's findings in the dogfish may be due to phylogenetic differences, however, activation of GP by Ca^{2+} alone may be relevant in the very early stages of burst exercise when ATP demand is extreme. Adrenergic activation of GP may allow for a continued supply of substrate for the later stages of the burst and for recovery purposes.

PFK phosphorylates fructose-6-phosphate (F6P) to fructose-1, 6-bisphosphate (F1, 6BP) and this reaction is essentially irreversible in vivo making it an excellent point of regulation. PFK is acutely sensitive to the energy status of the cell and is activated by AMP and ADP (i.e. when the energy charge is low) and is inhibited by ATP and citrate (i.e. when energy generation is adequate). Sensitivity to changes in AMP concentration is thought to be increased by substrate cycling between F6P and F1, 6BP. Thus small changes in ATP lead to proportionately larger changes in AMP and therefore a greater stimulation of glycolysis (Johnston, 1981). PK is regulated in a similar way to PFK in that it is inhibited at high ATP concentration and is activated by F1, 6BP.

1.4 Burst Exercise – physiological relevance

At high swimming speeds or periods of burst activity, large amounts of energy in the form of ATP is required. As the demand for energy goes up, breakdown of ATP leads to an increase in the level of ADP that shifts the mass action ratio of the creatine phosphokinase (CPK) reaction toward ATP production. PCr is catabolised by creatine phosphokinase to release the high-energy phosphate associated with creatine, (i.e. generates ATP from ADP) (Moyes & West 1995).

Overall, there is a net decrease in PCr levels (up to 90% depletion; Milligan 1996)) and release of P_i. As described above, endogenous ATP and PCr can only supply energy for a few seconds. Because the WM is poorly perfused, oxygen supply is low even during normal activity and the high power output of WM exceeds the capacity of mitochondrial (aerobic) metabolism (Moyes & West 1995). The large amounts of energy needed, therefore, must be supplied by anaerobic glycolysis since the WM is the first to become hypoxic when oxygen demand is high. Anaerobic metabolism is inefficient when compared with aerobic metabolism (only 2 moles of ATP are made from every mole of glucose, compared with 36 moles ATP for aerobic metabolism; Mathews & van Holde 1990). However, the rate of energy delivery by anaerobic glycolysis is much higher. Two end products accumulate during anaerobic glycolysis: lactate anions (formed by glycolysis per se) and protons (formed mainly in ATP hydrolysis, Hochachka 1985). Large amounts of lactate can be produced, lowering the pH of the muscle to ~ 6.7-6.8 (a typical resting pH measured in these studies being 7.0-7.2, Milligan 1996). The pH of the blood also decreases due to lactacidosis (metabolic acidosis) and increased production of CO_2 (respiratory acidosis). However, the magnitude of the pH decrease is not as great as that occurring in the WM. Due to the blood becoming acidified the level of oxygen saturation in the blood is reduced (Root effect). Maximum swimming speeds can only be maintained for a relatively short period with ~50% of glycogen being depleted in 15 s (Johnston 1981). This is possibly, in part, a regulatory mechanism that ensures that physiological disturbances are limited to the point where recovery is still possible.

1.5 The physiological significance of peri-mortem fatigue

The WM of fish is somewhat analogous to a battery. A finite amount of energy is stored in the WM (as in a battery) in the form of PCr, ATP and glycogen. If a fish has been fatigued prior to death most of its WM energy reserves in its battery will have been drained. As described previously, once the PCr reserves have been depleted and ATP levels cannot be defended, glycolysis is stimulated with supply of ATP being achieved via anaerobic glycolysis. This results in production of lactic acid that disrupts the acid-base balance in the fish, in turn lowering the pH of the WM. Other end products of ATP hydrolysis accumulate in the cells including P_i, CO_2 and creatine. Because the WM of fish is so poorly perfused, removal of waste products via the circulatory system is limited. Once the fish is killed and the heart stops beating, oxygen supply to the muscle is terminated. With no circulation the physiological and biochemical disturbances to the muscle are essentially 'locked' in place. If the level of physiological and biochemical disturbance in the WM is high the demand for ATP will also be high as the cells attempt to return to homeostasis. Thus, during PM storage the rate of dephosphorylation of ATP can be regarded as the primary reaction determining the rate of other chemical changes (Bate-Smith & Bendall 1956).

Development of rigor mortis during PM storage is dependent on the disappearance of ATP from the muscle (as described above). The rate of rigor development is largely determined by the immediate PM magnitude of the endogenous stores of ATP, PCr and glycogen in the WM. These sources of ATP together maybe looked upon as the "ATP potential" (term introduced by Bate-Smith & Bendall 1956). Thus, the PM stages can occur very quickly depending on the size of the ATP potential and the rate at which it is depleted. As stated above, the greater the demand for ATP the greater the reaction rate of other chemical changes, i.e. this hastens the onset of rigor mortis. In mammalian studies Bate-Smith & Bendall (1956) were one of the first groups to report the PM metabolic differences in psoas muscle of rabbits that either had a "quiet" death or a more "violent" death. They found that in the case of a quiet death ATP, PCr and pH values were high and PM changes were prolonged. In a violent death there was rapid disappearance of ATP and PCr. Because fatigue has a major impact on

the progression of PM changes in the WM it is of interest to evaluate how different capture and killing methods affect the physiological state of WM.

1.5.1 Capture method

The majority of fish capture methods used in the world today result in the fish being fatigued prior to leaving their habitat. Fishing methods such as trawling, set-netting and long-lining are so severe that fish are often dead before they leave the water. During trawling all fish in the path of the net are dragged along for various periods of time, (often over 2 h), and then the net is hauled aboard the vessel. Some fish that are caught late in the tow will still be alive, however, many will be dead due to exhaustion (trying to escape) and physical crushing if a large number of fish have been caught. Law & Jerrett (1997, unpublished results) showed that ~50% of pair-trawled snapper (Pagrus auratus) harvested live may not survive if released and only ~30% would survive long term in tanks (longer than 1 month). When fish are caught in a set-net the fish struggle resulting in fatigue. In a study by Chopin et al. (1996), 40% of sea bream (Pagrus major) captured in a trammel net died after being released. Of those that died, 28% of deaths occurred in the net due to the gill cover being held closed by the net. In the same study sea bream were also captured by hook and line. Using this method there were no mortalities during the 38-day post release period. Cortisol levels in the blood (a measure of stress) increased with increased capture time above control levels, indicating that fish struggled during capture. Capture of fish by the hook and line method is classified as a "passive" fishing and is the method preferred for the recovery of live fish. However, a study by Law et al. (1997) showed that the exercise resulting from capture of wild snapper by long-line may deplete all of the fishes aerobic energy reserves and ~80% of the anaerobic WM resources. Capture of muskellunge (Esox masquinongy) by angling (hook and line) resulted in acidification of the blood that took 12 to 18 h to recover, and also resulted in 30% mortality (Beggs et al. 1980).

All these capture methods result in the fish being fatigued due to the fish trying to escape (burst exercise). They also result in fish being physically damaged, e.g. scale loss, bruising and crushing. Even using a method such as potting in which fish swim into the pot via an opening and can swim round freely once inside, fish try to escape when the pot is being lifted. When the pot leaves the water the fish struggle on emersion and become fatigued (Black et al. 2001). On modification of the pot, whereby

the fish remain in a reservoir of water as the pot is lifted out of the sea, fish still struggle and become fatigued. While some methods of capturing fish are "better" than others, as far as survivability is concerned, they still fatigue the fish.

1.5.2 Killing method

There are many different ways to kill fish and due to the difficulty in handling live fish most of the methods result in the fish being further exhausted after the initial fatigue of capture. One of the oldest ways of killing fish is by suffocation. The fish is removed from the water and left in air whereby the gills collapse restricting the amount of oxygen uptake by the fish and eventually the fish dies. However, the fish normally struggles during this time and becomes fatigued thus hastening rigor mortis. A similar method for killing fish is by carbon dioxide (CO_2) anaesthesia. CO_2 is dissolved in water making it acidic. The fish blood also becomes acidic to levels below that normally experienced by the fish (even during exhaustive exercise), which eventually disrupts brain function and leads to death (Robb 2001). This method also results in the fish burst exercising during the procedure leading to depletion of energy reserves.

Low temperature killing involves fish being cooled to below their lower lethal limit. The fish become increasingly slower in their movements and a deep narcosis results (Ross & Ross 1999). However, the acute temperature drop that the fish experience when introduced to low temperature water (ice slurry) results in an escape response (burst exercise) as occurred with the two previous techniques.

Some fish such as tuna and salmon need to be bled prior to processing and this is often used as the method for killing the fish, i.e. fish die due to excessive blood loss. An extremely barbaric killing method is that of evisceration whereby the viscera is removed from the live fish resulting in blood loss. Many wild-caught fish are killed in this way; however, flat fish can take over 6 h to die after evisceration (Robb 2001).

Electrical stunning of fish has been trialed due to its wide use in poultry and red meat animals. Fish can be stunned quickly but there can be extensive damage to the carcass including haemorrhages and broken vertebrae (Robb 2001; Ross & Ross 1999). The rapid contraction of the WM during this procedure also results in fatigue in the WM.

Destroying the brain by inserting a sharp spike through the skull is a very rapid way of killing fish, however, requires accuracy for success. A similar method involves striking the head of the fish whereby the fish is stunned due to brain damage (percussion) and is instantly insensible. Quickly killing fish after capture has been shown to increase the pre-rigor period and also reduce the rate of lactate accumulation, flesh pH, ATP, PCr, and glycogen degradation in the WM compared with fish that were slurried or left in air (Amano et al. 1953; Azam et al. 1989; Boyd et al. 1984; Lowe et al. 1993; Mochizuki & Sato 1994; Nakayama et al. 1996; Nakayama et al. 1997). This practice highlighted the importance of having ATP still present in the WM immediately PM for extension of the pre-rigor period.

All the capture and killing methods outlined above result in the fish being fatigued prior to death. The following section addresses the physiological effect peri-mortem fatigue has on the WM and other PM factors that can either hasten or retard the progression of rigor mortis.

Several studies carried out on teleost species have now shown that rested or low-fatigue harvesting of fish can also result in significant improvements in post-harvest flesh quality (Boyd et al. 1984; Jerrett et al. 1996, 1998; Law et al. 1997; Lowe et al. 1993; Thomas et al. 1999; Wells 1987). It has also been noted that a stronger rigor tension develops in fish that have been highly active prior to slaughter than those that have not (Korhonen et al. 1990). This is thought to be due to the rapid rate of ATP depletion during exercise in comparison with rested fish (Berg et al. 1997).

1.6 The influence of temperature on the progression of rigor in the white muscle

Overall, peri-mortem fatigue is the overriding factor that will adversely affect the rate of PM changes in the WM. However, other factors such as storage temperature and acclimated temperature of the fish can also have a strong influence on the rate of progression through PM changes, either by hastening the changes or retarding them.

1.6.1 Storage temperature

To slow the progression through the PM stages in the WM, traditionally the strategy has been to chill the tissue rapidly with ice and maintain it at that temperature. With fish

that have been fatigued prior to slaughter, e.g. due to capture method (trawling, netting etc.) and ante-mortem handling, this is probably the best that can be done for the WM to delay the onset of autolysis. However, as the physiological state of the WM was different using the quick-kill method (ATP still present) several studies then investigated the effect that storage temperature had on the PM rundown of the WM. Three studies by Iwamoto et al. (1985, 1987, 1990), investigated the effects of storage temperature (0 °C and 10 °C) on development of rigor mortis in plaice, sea bream, yellowtail, bartailed flathead and Japanese striped knifejaw, that were dip-netted and pithed. They found that storage at 10 °C slowed degradation of ATP and onset of rigor mortis considerably. A comment should be made here that these studies had very limited replicates, and in one case, only one fish was stored at each storage temperature.

Something the papers by Iwamoto et al. did not mention was the effect cold shock had on the rate of onset of rigor mortis, instead they described the greater rate of metabolism at 0 °C simply as accelerated rigor. Parry et al. (1987) commented "the distinction between cold shock and rigor mortis has been made because the stiffening of some fish following icing appears to occur too soon after death to be consistent with the usual relationship between time of onset of rigor and temperature". Parry et al. found that tropical bighead (Aristychthys nobilis) acclimated to ~30 °C developed a level of stiffness much greater than normal rigor stiffness when stored at cooler temperatures (0-11 °C) compared with storage at higher temperatures (15 and 30 °C). Similarly, Curran et al. (1986) demonstrated that tilapia (Oreochromis aureus/niloticua hybrid, a tropical freshwater species) experienced a cold shock reaction such that they stiffened within minutes of being placed on ice. It would seem that the observation by Iwamoto et al. (1985) of immediate onset of rigor development of sea bream held at 0 °C was, in fact, cold shock.

In some species, such as chinook salmon (Oncorhynchus tshawytscha), PM storage of the WM down to 0 °C does not accelerate the progression of rigor mortis (Jerrett et al. 1998). The response was primarily dependent on the peri-mortem fatigue state of the animal. The optimum PM storage temperature for rested WM as described by the time to onset of rigor contraction was 0 to 4 °C. However, in partially fatigued muscle, temperature (0 to 12 °C) did not alter the time to onset of rigor contraction.

1.6.2 Acclimation temperature

Interestingly, the investigation carried out by Curran et al. (1986) contrasted the effects of storage of tilapia at 0 and 22 °C with common carp (Cyprinus carpio), a temperate freshwater species. Carp did not show stiffening due to cold shock at 0 °C and only went into rigor after ~16 h at both storage temperatures. Parry et al. (1987) made a similar comparison by contrasting bighead (acclimated to ~30 °C) and rainbow trout (acclimated to 4-6 °C). Rainbow trout showed no tendency to rapidly stiffen even when chilled to 0 °C. These two studies were probably the first to document that there were optimum storage temperatures for different species of fish acclimated to different temperatures. The significance of this finding is pertinent to the current study and will be discussed further in Chapters 3 and 7. Following on from this research came several studies investigating the effect acclimated temperature had on the development of rigor mortis. Environmental temperature is known to have large effects on metabolic rates, enzyme functions and structures, and changes to biological membranes (Blier & Guderley 1993; Guderley & Johnston 1996; Trigari et al. 1992; Wodtke 1981). Thus experiments were carried out to take advantage of the possible physiological short-term changes that would occur when fish were acclimated to different temperatures and how this would affect the WM PM. Hwang et al. (1991) and Watabe et al. (1990) both found that rigor mortis in carp progressed faster in fish acclimated to 30 °C than fish acclimated to 10 °C when stored at 0 °C after decapitation. Hwang et al. also showed that storage of WM closer to its acclimated temperature slows the onset of rigor mortis. This observation was taken a step further by Abe & Okuma (1991) who found that differences in storage and acclimation temperature between 0 and 5 °C delayed rigor mortis the longest (72 h) compared with tissue that had a 10 to 30 °C difference in storage and acclimated temperature (developed rigor mortis after 56 h and 24 h respectively). Two studies have made comments on the pre-mortem condition of fish and how it may affect post-mortem changes. Watabe et al. (1990) reported "the onset of rigor mortis and the period required to attain maximum value of the rigor index varied considerably depending on rearing conditions such as the tank size and starvation." Similarly, Curran et al. (1986) comparing cold shock in carp and tilapia, observed that PM levels of ATP in tilapia were considerably lower than in carp, and that the tilapia were more active or struggled more prior to death.

In studies carried out on rested chinook salmon acclimated to summer and winter temperatures, there was no difference in optimum PM storage temperature (0 to 2 °C; Jerrett et al. 2000). However, winter acclimated WM was 2.2 times more sensitive to temperature change. On the other hand, snapper (P. auratus) acclimated to summer and winter temperatures have quite distinct optimum PM storage temperatures for rested WM, corresponding to half the acclimated temperature (Jerrett et al. 2002).

1.7 Measurement of the physical changes to post-mortem white muscle

Being able to assess the PM status of the WM and/or follow the progression of rigor mortis has allowed researchers to gather information about how a stressor (e.g. capture method, storage temperature) affects the PM changes. Over the years there have been many methods developed to measure rigor and the progression of PM changes, some very simple, e.g. tail-sag method, and some very complicated, e.g. K-value. A brief review of some of these methods has been made for the reader to appreciate the advantages and disadvantages of such methods.

1.7.1 K-value

Due to ATP levels in the WM being implicated with the onset of rigor mortis, an indirect method of measurement was developed. This involves the measurement of WM ATP and its breakdown products (ADP, AMP, IMP, inosine (HxR) and hypoxanthine (Hx)), with the K value (or "freshness") being defined by the following equation described by Saito et al. (1959):

$$\text{K value (\%)} = \frac{[\text{HxR}] + [\text{Hx}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{HxR}] + [\text{Hx}]} \times 100\ \%$$

This method is very time consuming and requires expensive equipment, such as a high-performance liquid chromatograph (HPLC). It also involves complex extraction protocols, in which the high-energy phosphates critical to the measurement can breakdown, as they are highly labile. In WM with high levels of ATP (i.e. rested fish) the K-value does not resolve the pre-rigor period as the calculation is very close to 0%.

It is only when the ATP is hydrolysed and broken down to its end products that the K value registers a change.

Another biochemical measurement for following the progression of PM changes in the WM is the measurement of ATP and IMP spectrophotometrically with the absorbance expressed as a ratio. Any ratio over 1.0 means that there is more ATP in the muscle sample than IMP. Again, due to the labile nature of the metabolites being measured, the extraction procedure may result in an under estimate of the amount of ATP originally in the excised muscle.

1.7.2 White muscle pH

A relatively quick quantitative method was developed to measure the pH of the WM, whereby a muscle slurry is prepared and diluted with distilled water and then measured with a pH probe. Again, during the homogenisation of the muscle lactate can be produced which would lower the pH resulting in underestimation of the actual muscle pH.

1.7.3 Rigor index

One of the more simple methods for directly measuring the physical change in the fish carcass was that developed by Bito et al. (1983), a modification of the Cutting's method. This involves the fish being killed by destruction of the motor nerve (pithing) and then the anterior half of the fish body is placed on a board, suspending the posterior half free in the air. The distance between the level of the board and the base of the tail is measured by a rectangle scale at a certain time interval. The rigor index is expressed from the readings of the sag of the tail by the formula

$$\frac{L - L'}{L} \times 100\ \%$$

Where L is the sag value measured immediately after killing, and L' is the sag value at a certain time during storage.

Although this is a very simple, direct method of measuring rigor mortis it does not allow adequate characterization of the WM immediately post-harvest. Changes in the measurement only occur once the carcass starts to stiffen due to the onset of rigor mortis. A 0% rigor index only denotes the baseline for the group of fish being sampled

and cannot be compared with fish that have a different pre-harvest history. It is not a quantitative test as it only shows when there is a change in the "sag" of the tail, i.e. it is qualitative.

1.7.4 Muscle contraction force

An even more direct measurement of the physical changes in the tissue is to measure the force of the contracting muscle through the storage period. Strips of muscle are excised from the fillet and are attached to an apparatus (called a "rigorometer") that allows measurement of the contraction (Korhonen et al. 1990; Nakayama et al. 1997). This is (like the "tail sag" method) a qualitative rather than quantitative measure if the experimenter is not precise on the sampling site from the fish when excising the WM, and the dimensions of the strips are not consistent. It also does not characterise the pre-rigor period, as there is no contraction in the muscle.

1.7.5 Apparent failure stress

Another way of measuring the physical properties of the WM during PM storage is to measure the strength of the tissue, since muscle fibres become weaker with the onset of rigor mortis. This involves excising a strip of WM from a fish and then attaching both ends to an apparatus that can break the tissue strip by pulling it apart (see Jerrett et al. 1996). The force required to "break" the tissue, called the "apparent failure stress", is a quantitative measure of how strong the muscle is. It is called "apparent failure stress" as the WM strip is not a homogeneous block. There are distinct muscle blocks connected by myocommata and as rigor develops, failure of the muscle strips moves from a tearing failure (through the muscle block) to sharp failure (muscle blocks separate along the myocommata). This method is very descriptive of the strength of the muscle during the pre-rigor period. Once the WM is gaping (muscle block separation at the myocommata) and autolysing the test is limited as the tissue is too weak to measure.

There are several other methods for measuring rigor and an excellent review of the topic was made by Erikson (2001).

1.8 Inherent problems of experimentation using live fish

The concept of having "rested" fish as a control for experimental purposes is not new. Most of the literature investigating recovery from exhaustive exercise report baseline, "pre-exercise" levels of metabolites from "rested" animals. As mentioned earlier, handling of active fish is difficult and levels of high-energy phosphagens, such as ATP and PCr, can be depleted in a few seconds. Thus, a few flaps of a fish's tail can greatly reduce the levels of these metabolites in the WM with concomitant activation of anaerobic glycolysis, production of lactate and release of catecholamines into the circulation, i.e. a physiological state far from resting. In the past, the best experimenters have been able to do to minimise handling artefacts was to quickly capture fish (usually by dip-net) and kill them by putting a spike through the brain (pithing) or by percussion (sharp blow to the head, e.g. Thomas et al. 1999). Therefore, reporting this as the "unstressed" or "rested" state is not strictly true, due to the lack of control over capture and killing methods. Some experimenters take it a step further and allow fish to "recover" from capture stress. Before recovery the fish are often cannulated for further blood sampling and this surgery is usually performed under light anaesthesia, using the inhalation anaesthetic MS-222 (ethyl *m*-aminobenzoate methanesulphonate). Following such procedures, the fish is then allowed to "recover" for a nominal period (typically 1-3 days) before further experimentation begins (Milligan and McDonald, 1988; Wang et al. 1994; Waring, et al. 1996). In theory, this sounds reasonably acceptable, as the recovery period would allow the fish to regain metabolic and biochemical homeostasis. However, the fish is usually contained in a "black box" set-up whereby water enters and exits a long narrow box in a flow-thru manner at a velocity fast enough that the fish can swim against it. Again, this sounds reasonable, however, even though the fish may not be struggling it maybe suffering from chronic stress due to its unfamiliar environment, and any trauma associated with cannulation. Classing animals as unstressed or rested becomes a matter of opinion but can be characterised by analysing key indicators of fatigue such as blood lactate, glucose and catecholamines levels. Any results obtained after overlaying further stressors in the course experimentation, e.g. storage temperature, must then be eyed with caution due to a lack of characterisation of muscle before the experiment began.

Measurement of key indicators of fatigue, such as blood and muscle lactate, muscle ATP, blood pH, and plasma catecholamines levels give a good indication of physiological condition. Wells (1987) commented that food technologists were obtaining baseline values of metabolites from fresh fish without taking into account the physiological disturbance of capture. It is difficult to evaluate the real advantages of strategies for delaying the onset of rigor mortis without knowing what the "true" resting metabolite levels are, and the impact of levels, other than resting ones, may have on the PM rundown of the WM.

The development of a non-destructive, non-invasive technique to measure muscle metabolites has gone some way in being able to report "resting" in vivo levels. One such method is ^{31}P nuclear magnetic resonance (NMR). This measures phosphorus-containing compounds such as sugar phosphates, P_i, IMP, phosphodiesters, PCr, and ATP (van den Thillart & van Raaij 1995). From these measurements intracellular pH can be calculated. Typical intracellular pH levels of fish in normoxic conditions have been reported as 7.36 ± 0.05 for goldfish, and 7.36 ± 0.04 for carp. These values are higher than those reported for "rested", quickly killed fish, however, the fish still have to be restrained for measurement. In a study by van Waarde et al. (1990) fish were kept in a darkened tank overnight and then anaesthetized with MS-222 (3-aminobenzoate ethyl ester methanesulphonate) and mounted in a flow cell of the in vivo NMR probe. During experimentation fish were immobilized by being pressed flat against the side of the flow through cell by an inflatable plastic bag. No anaesthetic was present in the water of the cell and therefore the fish woke rapidly and was conscious during the experiment. One of the drawbacks of this method is the cost of the equipment which is extremely expensive and out of reach of most laboratories. Also, the fact that the fish was aware during experimentation casts doubts on how rested the animal was during measurements.

The use of anaesthetics for aquatic animals has been widespread. Concentrations of anaesthetics to enable sedation of the animal are used routinely for length-weight measurements, sexing, simple injections, withdrawal of body fluids, etc. However, full surgical anaesthesia maybe required for some procedures (Ross & Ross 1999). Most anaesthetics used routinely are of the inhalation type where the anaesthetic is added to the water in which the fish are swimming in and is taken up through the gills. As some of these chemicals are very expensive, only small amounts are used and

animals are often dip-netted out of their rearing tank and into a bin containing water and the anaesthetic. This somewhat defeats the purpose of having fish that are rested if they have to be chased around with a dip-net and then struggle in the capture process. It maybe termed the "unstressed" control group of fish, compared with the way "stressed" fish are harvested, but again, the lack of control over the capture and handling of the fish may lead to inconclusive results during experimentation.

Due to the difficulty in working with live fish Jerrett et al. (1996) commented that the post-harvest characteristics of "rested" fish muscle may not have been fully described and warranted further investigation. Their objective was to study the impact of two specific states of muscle exhaustion (rested vs. exhausted) on the PM tensile properties of the WM of cultured chinook salmon (Oncorhynchus tshawytscha). Fish were rested for 20-24 h in their rearing tank prior to introduction of the food grade anaesthetic, AQUI-S^{TM} (AQUI-S NZ Ltd, Lower Hutt, New Zealand) into the tank (i.e. fish were not removed from their home tank into a smaller volume). The fish did not react adversely to the anaesthetic and over a period of ~30 min the depth of anaesthesia reached was deemed suitable for further handling (loss of equilibrium, weak swimming motions, insensible to forced extension of the gill operculum and contact with the gill lamellae). Fish were then killed by pithing using a traditional Japanese ike jime tool. Within 3 min of pithing a cut surface pH measurement of the WM was taken (measurement was made on the surface exposed by the transverse section of the fillet). The mean resting pH was 7.38 ± 0.05, (± SEM), compared with 6.45 ± 0.06 for WM that had been exhausted by a controlled protocol of electrical stimulation. It was suggested that the mean "resting" pH was close to its true resting state given that it was slightly higher than measurements of intracellular striated muscle pH from other poikilothermic species (bullfrog and turtles) at similar temperatures.

In this experiment it was found that after 40 h storage at 2 °C the apparent failure strength (described in Section 1.7.5) of "rested" chinook salmon WM was 2.7 times that of the "exhausted" muscle with the "rested" muscle retaining its immediate post-capture strength. Interestingly enough, "rested" carcasses, after ~20 h storage, were still electrically excitable even though the carcass was rigid. Jerrett et al. (1996) commented that this illustrated the unreliability of extrapolating from the state of rigor mortis as measured by the modified "Cuttings method" or "Rigor Index", (Bito et al. 1983) to specific tissues (see Section 1.7 : Measurement of the physical changes to the

WM post-mortem). A similar case was also seen in the study by Lowe et al. (1993) where Rigor Index of rested fish increased dramatically during storage at 3 °C in an ice-seawater slurry (i.e. fish stiffened). However, the K–value of rested fish barely changed, suggesting that ATP levels were still high even though the Rigor Index was high.

1.9 The present study

The development of aquatic anaesthetic AQUI-S™ and the subsequent studies by Jerrett & Holland (1998) and Jerrett et al. (1996, 1998, 2000) highlighted the importance of reducing peri-mortem fatigue on PM metabolism of chinook salmon. Being able to biochemically and physically characterise WM from fish as close to true resting levels as possible also highlighted that the muscle could be in a state post-harvest that had never been seen or characterised before. As this was carried out in chinook salmon it was thought that it would be pertinent to extend the research to other commercially important species in New Zealand due to the fact that most teleost species have a true WM rather than the mosaic structure seen in salmonids. Thus the rested WM of two New Zealand teleost species as well as chinook salmon were biochemically characterised.

With the exception of measuring apparent failure stress of the WM PM, the other methods are only descriptive of the changes occurring once the WM starts to become depleted of ATP and enters rigor mortis. The focus of this study is on extending the pre-rigor period, a period that has been poorly charaterised in fish WM because of the rapid biochemical changes that can occur hastening the onset of rigor mortis. It was my aim to further characterise this period and therefore the methods outlined above were of little use. I have concentrated on measuring specific biochemical changes in the WM as indicated by changes in key metabolites, i.e. lactate, ATP, P_i creatine and glycogen, as well as WM pH. Together, the changes occurring in the WM indicate the ability of the PM tissue to defend ATP levels.

Because the rested harvested muscle retained most, if not all of its endogenous fuel stores (i.e. had a high ATP potential) the options for extending the pre-rigor period by further retarding the demand for ATP were investigated.

As WM ATP is most efficiently maintained by aerobic metabolism some of the focus also turned to the cellular level, in particular to the mitochondria (the site of oxidative phosphorylation). Due to the WM being mainly anaerobic in function during burst exercise, but knowing that at rest and during recovery from exercise the muscle is probably maintained aerobically, it was important to determine factors that could possibly alter or inhibit the normal functioning of the mitochondria in the WM. It is known that during anoxia mitochondria change from being ATP producers to potentially powerful ATP consumers, a phenomenon known as "cellular treason" (St-Pierre et al. 2000).

The concept of keeping isolated tissue alive, i.e. ischemic preparations, is not something that is new to the medical world. Organ preservation has become commonplace, and medical science has come a long way in extending the period certain donor organs/tissues can remain ischemic but still remain viable for transplantation purposes. Although this is accepted in medical science, similar research has not been carried out on foodstuffs that are muscle based in order to extend their "fresh" storage life.

The primary objective of this thesis was to investigate how cell viability can be maintained in rested ischemic fish WM during PM storage. Species differences were examined as well as environmental factors to uncover correlates with PM metabolism.

Chapter 2 outlines the general methods and materials used throughout the thesis.

Chapter 3 characterises the ischemic WM of yellow-eye mullet (Aldrichetta forsteri) during PM storage by measuring biochemical indices. Rested harvesting techniques result in WM that is physiologically and biochemically consistent (low variation in measurements), with energy stores intact. The consistency of the WM allowed the author to accurately characterise how the rested WM "behaved" during PM storage. This was essential before attempting any manipulation to extend the "life" of the ischemic WM. Factors such as fish size, age and acclimation temperature were also investigated as to their effects (if any) on PM metabolism.

Chapter 4 specifically looks at retarding the PM changes in the WM of yellow-eye mullet. The ischemic WM preparation is limited to anaerobic energy generation by its very nature (i.e. no circulatory system). The low efficiency of anaerobic glycolysis and

the acidification that results limits how long ATP can be generated PM. As aerobic glycolysis is far more efficient at ATP generation an attempt was made to deliver the limiting substrate (i.e. oxygen) to the ischemic WM preparation during PM storage. This was achieved using hyperbaric techniques, not dissimilar in theory to those used for hyperbaric oxygen (HBO) treatment in humans. PM storage under these conditions resulted in significant retardation of the rate of acidification and biochemical changes in the WM. Physical parameters such as pressure, flow and temperature were also assessed as to their influence on the PM changes. A standard storage protocol was devised and a detailed characterisation of the WM during PM storage was made. A period of aerobic ATP generation in the first 12 h of PM hyperbaric storage was evident.

Chapter 5 applies the methods used in Chapter 4 to two commercially important species of fish: snapper (Pagrus auratus), a temperate sparid, and chinook salmon (Oncorhynchus tshawytscha), a salmonid. Species differences were observed during PM storage of the WM under normobaric and hyperbaric conditions. The PM differences in the WM were attributed to the differences in muscle structure and their possible differing biochemical strategies for recovery from burst exercise. However, physical condition of the fish also played a major role in the progression of PM changes in the WM.

Chapter 6 looks into the reasons why cell viability is still lost even though the essential substrates (glycogen and oxygen) are available to the WM during hyperbaric storage. The focus was at the cellular level, in particular the mitochondria, the site of oxidative phosphorylation. Factors such as pH and CO_2 were investigated to determine their effects on respiration of the mitochondria. The investigation was made to determine if acidification alone halts aerobic glycolysis or is it due to a combination of factors. Extraction of viable mitochondria from the WM during PM storage under hyperbaric and normobaric conditions identified the point at which aerobic respiration ceased in both preparations. The behaviour of the mitochondria under normobaric and hyperbaric storage differed somewhat and reasons for that are discussed.

Chapter 7 focuses on the effect acclimation temperature has on the PM metabolism of ischemic WM held under hyperbaric conditions. The results of Chapter 3 showed that there was no difference in PM metabolism in winter and summer acclimated WM held

under normobaric conditions. However, storage under hyperbaric conditions uncovered some interesting differences. The results suggest that summer acclimated mullet WM has a larger aerobic scope than winter acclimated fish.

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CHAPTER 2

General materials and methods

This chapter outlines the General Materials and Methods used in the thesis. Specific details applying only to a particular chapter are included in the Materials and Methods section of that chapter.

2.1 Experimental animals

2.1.1 Yellow-eye mullet

Yellow-eye mullet (Aldrichetta forsteri) were captured from Nelson Harbour in November and December 1996 and 1998, and also September and October 2001 using a modified snapper trap. The trap was set from the wharf extending from the Seafood Research Unit where the laboratory is situated. The fish usually showed signs of capture injury (skin lesions) several days after being caught and were treated with malachite green and formalin (25 ppm in seawater, exposed for ~1 h) to help heal lesions. Mullet were reared indoors in round black PVC tanks (1.82 m^3, 1.41 m dia.; Model: Roto Open Top Black Skellerup Industries Ltd, Christchurch, New Zealand). Each tank was supplied with auxiliary aeration to maintain the dissolved oxygen concentration at 80-90% saturation. The volume of seawater in the tank was ~1000 L and was supplied with sand-filtered seawater pumped from the Nelson Harbour on a flow-through basis (10 L/min). During rearing, the fish were exposed to natural photoperiod and ambient seawater temperatures. Fish were minimally disturbed for daily feeding and weekly cleaning.

2.1.2 Snapper

Juvenile female snapper (Pagrus auratus) ~9 g in weight were obtained from the National Institute for Water and Atmospheric Research (Mahunga Bay, New Zealand) in October 1994. These snapper were first generation fish bred in captivity from wild Tasman Bay (New Zealand) stock. Snapper were reared indoors in 6.08 m^3 (2.7 m diameter) tanks supplied with sand-filtered seawater pumped from the Nelson Haven on a flow-through basis (30 L/min). During rearing, the fish were exposed to natural

photoperiod and ambient seawater temperatures. Snapper were sampled in December 1998.

2.1.3 Chinook salmon

Female chinook salmon, (Oncorhynchus tshawytscha), ~80 g in weight (smolt) were obtained from the New Zealand King Salmon Co. Ltd. Hatchery (Waikaropupu Springs, Golden Bay, New Zealand) in September 1997. Salmon were reared indoors in a 12 m^3 (4 m dia.) tanks supplied with sand-filtered seawater pumped from the Nelson Haven on a flow-through basis (30 L/min). During rearing, the fish were exposed to natural photoperiod and ambient seawater temperatures. However, during the summer months the influent water was cooled with a heat-exchanger (Model: 38QRS80-837 "Super Quiet", Carrier, Farmington, CT, USA) to ~18 °C as salmon do not naturally tolerate high ambient temperatures. Salmon were sampled in January 1999.

The rearing tanks for salmon and snapper were also surrounded by opaque PVC screens. These extended 1.2 m above the water level. This allowed workers to move about the tanks without being seen by the fish, preventing undue disturbance. A 0.8 m portion of the screen could be opened to allow daily feeding from the same position on the tank edge. The screens could also be removed for bi-weekly tank cleaning

2.2 Fish conditioning

Mullet and salmon were maintained on proprietary diets (NRM Ltd, Nelson, New Zealand) appropriate to their size. Feed was supplied at ~2-3% body weight per day for the salmon and ad libitum for the mullet. Snapper were fed primarily on an alginate-bound moist feed based on the diet reported by Spotte, (1992), and supplemented with a range of proprietary snapper diets (NRM Ltd, Nelson, New Zealand) appropriate to their size. Feed was supplied at 4.5% of their body weight per day due to the lower level of protein in the alginate-bound diet. All handling for routine measurement and husbandry was done under anaesthesia (AQUI-S™ Plus, AQUI-S New Zealand Ltd., Lower Hutt, New Zealand).

During rearing, the fish were exposed to natural photoperiod and ambient seawater temperatures. The Nelson Haven experiences relatively large seasonal fluctuations in sea temperature ranging from ~8 to 23 °C.

2.3 Harvesting method

Throughout this study all fish were harvested using the techniques of rested harvesting, with the exception being the fish that were exercised prior to harvest. The food grade anaesthetic AQUI-S™ Plus was used to anaesthetise fish without eliciting "startle" reflexes or increased swimming activity, producing rested fish. A 1:10 stock solution of anaesthetic/water was added to the rearing tank to achieve initial concentrations of between 17.0 ± 0.5 mg/L and 30.0 ± 0.5 mg/L, depending on the species being anaesthetised. To ensure adequate mixing, anaesthetic was introduced into the up-welling caused by aeration (experimental timing recorded from the time of introduction of anaesthetic to the tank). The fish were disturbed as little as possible during this procedure. After 5-15 min (depending on species) the fish would not avoid obstacles placed in their path. At this stage the fish were classed as sedated. After 25-40 min the fish became anaesthetised and exhibited loss of equilibrium, very weak swimming motions, slow operculation and were insensitive to forced extension of the gill operculum and contact with the gill lamellae. No burst swimming was observed during induction of anaesthesia.

The fish were deemed suitable for further processing when the depth of anaesthesia was determined to be approximately Stage IV to V (after Jolly et al. 1972). In the proceeding chapters there are more detailed accounts of the anaesthetisation process used for each experiment.

2.4 Blood sampling and pH, lactate and glucose measurement

A mixed venous blood sample (1 mL) was taken by cardiac puncture using a heparin rinsed syringe (Terumo 1 mL syringe, ½ inch needle, 26 gauge; rinsed with 0.04 g Li/heparin per 100 mL distilled water) once fish were anaesthetised and suitable for handling. The blood was transferred into 1.5 mL Eppendorf tubes. The pH of the blood was measured immediately after extraction using a combined pH electrode (Model: pHC 2406, Radiometer, Copenhagen, Denmark; calibrated as directed) connected to a pH/mV meter (Model: PHH92 Lab Meter, Radiometer, Copenhagen, Denmark). The electrode was directly inserted into the Eppendorf minimising pH changes associated with changing CO_2 content.

The lactate concentration in the blood was measured using an Accusport lactate meter (Model: 1488767, Boehringer Mannheim, Germany) set to measure whole blood. The glucose concentration of the blood was measured using an Advantage® Glucose Meter (Model: 800767, Roche Diagnostics, Auckland, New Zealand).

2.5 White muscle storage

After blood sampling the fish were pithed (brain and spinal cord ablation) using a traditional Japanese iki-jime tool. The fillets were excised from the carcass and then appropriate groups of fillets (detailed in each chapter) were placed on a rack, skin side down. The rack was made of 3 plastic grids (each grid 213 x 57 mm) spaced evenly. One fillet was placed on the bottom level, two in the middle and two on top. The rack was then placed into a clear plastic chamber (Model: W10PR Filterpure Housing, Microlene, USA) containing ~25 mL of Teleost Ringer's (Nilsson & Fange 1969) to prevent dehydration of the fillets. Inlet and outlet pipes (4 mm OD, Leda-thene Pneumatic Tube) for gas delivery (specific gases outlined in each chapter) were attached to the chamber and then the chamber was submerged horizontally (ensuring fillets lay flat), in a water bath (Model: LTD20G, Grant Instruments Ltd., Cambridge, England) at a specified temperature. The temperature accuracy of the waterbath was confirmed using a Precision Thermometer (Model: 4600, Yellow Springs Instruments Inc., Yellow Springs, Ohio). The filter housing was flushed with the appropriate gas for ~5 min. Prior to the gas entering the filter housing it was bubbled through a chamber of distilled water to humidify it to ~95% (measured with a relative humidity/air temperature sensor, Model: 1400-104, LI-COR Inc., Lincoln, NB, USA). The gas flow was set to 50 mL/min ± 1.25% (flow meter Model: C1D-PC, Platon Instrumentation, Basingstoke, England), unless otherwise stated. The flow meter was calibrated with a Mass Flo® Controller (Model: Type 1179A, MKS Instruments, Andover, MA, USA).

2.6 White muscle pH measurement

The cut-surface WM pH measurements and WM samples were taken from the WM of fish immediately after filleting and then at various times throughout the PM storage period. The pH measurements were made on the surface of the D1 muscle block (runs the length of the fillet, close to the vertebral column) exposed by a transverse section of the fillet as described by Jerrett et al. (1996) using a combination pH surface electrode

(Model: 450-C, Sensorex, Garden Grove, CA, USA; calibrated as directed) connected to a pH meter (Model: PHM 202 Lab Meter, Radiometer, Copenhagen, Denmark). The pH probe was calibrated at room temperature and then the temperature of the meter was set at the acclimated temperature of the fish.

At each sampling time a transverse slice was taken to expose a fresh section of WM on each fillet. The fillets were serially sampled throughout the experiment. Only thin slices of tissue (see Section 1.7) were taken for biochemical analysis. Care was also taken to only measure the pH of the D1 muscle block, which runs the length of the fillet. There was a concern that the biochemical parameters being measured in the WM (lactate, ATP, creatine, P_i, and glycogen) would vary as a function of position along the epaxial musculature since each fillet was serially sampled during PM storage. However, a study by Lowe (1992) found that there was little difference in total [ATP] from locations along the epaxial musculature of snapper (Pagrus auratus) apart from samples taken close to the tail. In the present study I avoided taking samples from the tail by limiting sample size and numbers, and it was not considered likely to have adversely affected the results. Further to this, a study by Somero & Childress (1980) investigating glycolytic enzymes in different sized teleosts found "no positional sampling artefacts" in enzyme activities along the length of the fish (Paralabax clathratus). Ideally, different fillets would have been used at each sampling time to avoid any artefacts associated with serially sampling, however, this was not possible due to the limitations of the storage vessel size.

2.7 White muscle sampling

A transverse slice of muscle (~3 mm thick) was taken from each fillet and immediately freeze-clamped between blocks of aluminium cooled in liquid nitrogen. This was a very rapid method for freezing the tissue, only requiring ~5 sec of clamping. The frozen sample was then wrapped in aluminium foil and stored in liquid nitrogen until extraction.

It is important when excising tissue for metabolite analysis that sampling artefacts are minimised. Once a tissue sample has been taken it must be metabolically inactivated as soon as possible. A study by Wang et al. (1994) investigating the effect of different sampling and processing methods on rainbow trout WM metabolites found

that overdose with MS-222 followed by freeze-clamping of excised muscle preserved higher levels of PCr and glycogen than the rapid needle biopsy method (fish are not anaesthetised). Van den Thillart & van Raaij (1995) also found that freeze-clamping was the most effective way to metabolically inactivate WM but careful tissue extraction methods were required.

2.8 Tissue extraction

A WM sample was removed from liquid nitrogen and placed in a ceramic mortar (7 cm diameter) containing liquid nitrogen. To obtain tissue specifically from the D1-block muscle the frozen sample was broken into smaller pieces with the pestle and tissue from the desired area was removed with forceps cooled in liquid nitrogen. A 0.10 g sample of this tissue was quickly weighed and returned to the cleaned mortar refilled with liquid nitrogen. The sample was then ground to a powder with the mortar being topped up with liquid nitrogen as needed. Once the sample was powdered and still under liquid nitrogen, 0.5 mL of 0.1 mol/L perchloric acid (PCA) containing 30% ethanol was added to the mortar (the ethanol acted as an anti-freeze agent; van den Thillart & van Raaij 1995). The sample and PCA solution were ground together to a powder then transferred quickly to an Eppendorf tube using a spatula cooled in liquid nitrogen. Immediately after transfer, the still frozen powder was homogenised using a hand-held Ultra-turrax (Model: T8: Ika Laboratechnik, Staufen, Germany) set on the highest speed for 30 sec. A 0.1 mL sample of the homogenate was then taken and set aside, on ice, for glycogen analysis (see Section 2.9.5). Homogenised samples were stored in ice and then centrifuged for 5 min at 10 000 rpm in a bench top centrifuge (Model: MSB010.CX2.5 MSE Micro-Centaur, Sanyo Gallenkamp PLC, England). The supernatant was retained and then neutralised with a known volume of 5 N potassium hydroxide and centrifuged again. The neutralised supernatant was then used in the metabolite determinations (excluding glycogen in which the whole homogenate was used).

Breaking up the frozen tissue slices in order to get the right amount of tissue for the extraction had proved difficult in the past due to a much larger piece of tissue being frozen for each sample. The sample would be crushed with a block of aluminium and 2 g of tissue would be extracted in PCA. This method resulted in partial thawing of the tissue during the crushing process and then being fully thawed when extracted. The method was scaled down and modified in the current study to avoid such problems.

2.9 Metabolite determinations

2.9.1 Lactate

Lactate content of the WM was measured using a portable blood lactate meter (Accusport Model 1488767, Boehringer Mannheim, Germany). The neutralised sample extract was used instead of whole blood or plasma with the meter set on plasma mode. A standard curve was constructed and readings converted accordingly (see Appendix 1).

Lactate was determined by reflectance photometry via a colourimetric lactate-oxidase mediator reaction on the test strip. Previously the lactate content of WM had been determined enzymatically using the Boehringer Mannheim analysis kit (Cat. No. 139 084). Because the enzymatic method was quite time-consuming, possibly resulting in artefacts in the measurements (tissue extracts not totally inactive through the enzymatic process) an alternative was found to measure lactate that required no further processing other then extraction of the tissue sample.

2.9.2 Adenosine triphosphate

ATP content of the WM was determined using an ultraviolet spectrophotometric analysis as for whole blood (Catalogue No. 366-A, Sigma Diagnostics, St. Louis, MO, USA). The sample extract was used instead of whole blood. The ATP assay was not specific for ATP, it read GTP, ITP and UTP as ATP. The ATP was assayed using phosphoglyceric phosphokinase (PGK) and glyceraldehyde phosphate dehydrogenase (GAPD) as follows:

$$\text{ATP} + \text{3-phosphoglycerate} \rightarrow \text{ADP} + \text{1,3 diphosphoglycerate}$$

$$\text{1,3 diphosphoglycerate} + \text{NADH} \rightarrow \text{glyceraldehyde-3-P} + \text{NAD} + P_i$$

The reduction in the amount of NADH present was stoichiometric to the amount of ATP in the sample. A standard curve was constructed to determine the concentration of ATP instead of using the calculations outlined in the Sigma method (see Appendix 1). The amount of sample in the assay was halved in order to give an absorbance reading in the linear part of the standard curve.

2.9.3 Inorganic phosphate

P_i content of the WM was determined using a modified ultraviolet spectrophotometric analysis for whole blood (Catalogue No. 670-A; Sigma Diagnostics, St. Louis, MO, USA). The sample extract was used instead of whole blood. The phosphate ions react with ammonium molybdate at acid pH, forming ammonium phosphomolybdate. Reaction of this compound produces a blue phosphomolybdenum complex:

$$\text{Phosphorus} + \text{Ammonium Molybdate} \xrightarrow{\text{Acid pH}} \text{Ammonium Phosphomolybdate}$$

$$\text{Ammonium Phosphomolybdate} + \text{Aminoaphyholsulfonic acid} \rightarrow \text{Heteropolymolybdenum (Blue)}$$

The ammonium molybdate solution was modified to 1.25 g/dL ammonium molybdate in 2.5 N H_2SO_4 then diluted by 10 prior to use. One mL of the diluted ammonium molybdate solution was mixed with 10 µL of neutralised extract in a 2.5 mL cuvette and then the absorbance was measured at 340 nm. A standard curve was constructed to determine concentration (see Appendix 1).

2.9.4 Creatine

Creatine content of the WM was determined using a spectrophotometric method described by Eggleton et al. (1943) that was modified so the assay could be performed in a 2.5 mL cuvette. The assay used was based on the chemical reaction:

$$\text{creatine} + \alpha\text{-naphthol} \rightarrow \text{coloured complex (pink)}$$

After scanning the coloured complex in the spectrophotometer it was found that the greatest absorbance occurred at 525 nm and thus all absorbance measurements were carried out at this wavelength. As the colour intensity of the assay was too great, a dilution of 10 times was required prior to measurement. Further investigation into this method revealed that the dilution step was performed at the wrong time. This meant that creatine values over 20 µmol/g fell outside the linear part of the calibration curve and were not reliable. For further discussion see the "Results" section of Chapter 3. A standard curve was constructed to determine concentration (see Appendix 1).

ATP, P_i and creatine absorbance measurements were made using a UV/Vis Spectrophotometer (Model: UV2-100; ATI Unicam, Cambridge, England).

2.9.5 Glycogen

Glycogen content of WM in mullet was determined using a modification of the method described by Keppler & Decker (1974). The glucose produced by the hydrolytic reaction was measured using a Reflotron® IV Analyser (Boehringer Mannheim, Germany).

In an Eppendorf tube 0.5 mL of amyloglucosidase solution (2 mg/mL in acetate buffer) containing an internal glucose standard (1 mmol/L) was added to 0.1 mL of homogenate along with 0.05 mL potassium hydrogen carbonate (1 mol/L). This was mixed and an initial reading was taken on the Reflotron®. The reaction taking place on the Reflotron® glucose test-strips was:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \delta\text{ - D – gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{indicator} \xrightarrow{\text{POD}} \text{dye} + H_2O$$

The mixture was then incubated with shaking at 40 °C for 2.5 h. Samples were then placed on ice to stop the reaction and a final glycogen reading was taken on the Reflotron®. A standard curve was constructed and the Reflotron® glycogen readings calculated accordingly (see Appendix 1).

2.10 Statistical analysis

Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows Version 6.00 (SPSS Inc.) and Microsoft® Excel 2000. In most cases when comparisons were made between groups of fish a Student's t-test was used. Although this may not have been statistically "correct" because of low degrees of freedom of the sample groups the Student's t-test is very powerful and robust. The differences between sample groups were usually large and variance was small. Therefore a decision was made to use a simple statistical test, such as the Student's t-test, to illustrate differences. To make comparisons between sample times during PM storage of samples the Sign-test was used (essentially a binomial test with p hypothesised to be 0.5; Zar 1984).

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CHAPTER 3

Post-mortem white muscle metabolism of rested and exercised yellow-eye mullet (Aldrichetta forsteri).

3.1 ABSTRACT

Peri-mortem fatigue has been identified as one of the major factors that reduces the time before the WM enters the PM state of rigor mortis. A comparison was made between the PM changes that occur in summer and winter acclimated rested mullet and winter acclimated exercised mullet WM. Fish were anaesthetised with AQUI-S^{TM} Plus in a rested state. Peri-mortem exercise resulted in decreased blood pH and increased blood lactate and glucose levels. Ischemic WM was stored at half the ambient temperature of the fish (optimum PM storage temperature) in chambers flushed with humidified pure oxygen (95% humidity) at a rate of 50 mL/min ± 1.25%. Measurement of cut-surface pH was used to follow the PM changes occurring in the WM. Rested mullet had much higher WM pH immediately after harvest compared with exercised mullet, 7.68 ± 0.02, (± SEM) and 7.14 ± 0.06, respectively. The rate of acidification in the exercised WM was ~2.6 times that of rested WM. During PM storage exercised WM pH reached a plateau of pH 6.4 after 15 h compared with 25 h in rested WM. There was no difference in PM WM pH profile between rested fish of different sizes (weight difference ~80 g). Biochemical and metabolic changes occurring in the rested WM during PM storage were characterised by measuring key metabolites (lactate, ATP, P_i, creatine, and glycogen). Pre-storage WM ATP and glycogen levels in rested mullet were high (7.4 ± 0.7 μmol/g and 37.6 ± 5.6 μmol/g, respectively) with low lactate, (12.7 ± 1.0 μmol/g), P_i (22.9 ± 2.1 μmol/g) and creatine (12.6 ± 1.0 μmol/g) concentration. This was attributed to the conservation of energy reserves by the rested harvesting method. ATP and glycogen levels were depleted over the first 25 h of storage along with a sharp rise in [lactate]. P_i and creatine levels also increased during storage. The buffering capacity of the rested WM and the mean standard oxygen consumption of rested and exercised, anaesthetised mullet were also measured and their significance to PM metabolism is discussed. The maintenance of the WM ATP and glycolytic substrate stores prior to

storage was critical in allowing the muscle cells to maintain homeostasis for an extended period prior to the onset of rigor mortis.

3.2 INTRODUCTION

Extensive research has been carried out describing the PM changes that occur in teleost WM during storage (see Chapter 1: General Introduction). However, understanding of how the physiological state of the WM immediately post-harvest affects the time course of the PM changes has been more of a recent discovery. In mammalian studies Bate-Smith & Bendall (1956) were one of the first groups to report the PM metabolic differences in psoas muscle of rabbits that had either a "quiet" death or a more "violent" death. They found that in the case of a quiet death ATP, PCr and pH values were high and PM changes were prolonged. In a violent death there was rapid disappearance of ATP and PCr. Several studies carried out on teleost species have now shown that rested or low-fatigue harvesting of fish can also result in significant improvements in post-harvest flesh quality (Boyd et al. 1984; Jerrett et al. 1996, 1998; Law et al. 1997; Lowe et al. 1993; Thomas et al. 1999; Wells 1987). Peri-mortem fatigue in teleosts has been identified as the major factor in reducing the time it takes the WM to enter the state of rigor mortis and deteriorate further due to autolysis (see Chapter 1).

The WM of teleosts is analogous to a battery with burst exercise rapidly draining the battery of stored energy. It is difficult to capture and handle live fish without the fish struggling. They are often fatigued during handling procedures, altering the physiological state of the WM even before experimentation begins. Thus few studies can report true "resting" levels of traditionally measured post-harvest parameters such as WM pH, ATP, lactate etc.

If the physiological state of the WM is disturbed during the peri-mortem period the metabolic artefacts associated with the disturbance make it difficult to discriminate between treatment effects and inherent variation in the starting material. Therefore, it is very difficult to accurately determine any scope for improvement if the results have been "clouded" due to fatigue artefacts. Consideration of how the peri-mortem physiological state of the WM may influence the parameters overlaid on the tissue during post-harvest storage is seldom emphasized. Jerrett et al. (1996) reported post-harvest metabolic parameters that were measured in Chinook salmon WM. Using rested harvesting techniques, together with conservative handling procedures, this was possibly the first time that such parameters had been reported as close to "resting" levels as possible. The PM changes in the metabolic parameters were followed during storage

and compared with WM that had been electrically exercised post-harvest. That study showed that increases in WM lactate levels did not occur until 20 h PM in the rested tissue, i.e. anaerobic glycolysis was not required to produce ATP via lactate production until this time. In contrast, exercised WM relied solely on anaerobic ATP generation during PM storage, hastening cell death. Although high-energy phosphates were not measured in this study the use of rested harvesting techniques would have conserved these energy reserves i.e. ATP and PCr, as suggested by the PM changes in WM lactate.

The Jerrett et al. (1996) study was carried out on chinook salmon, a species commercially important in New Zealand, but not typical of native, inshore teleosts. With increased awareness of the finite nature of fish stocks it has become increasingly important to ensure that we gain the maximum value from every fish caught. To this end it is necessary to determine the scope for improvement in other New Zealand commercial species. Thus, the objective of the study was to extend the PM metabolism knowledge gained in salmonids to a marine teleost species (yellow-eye mullet, Aldrichetta forsteri; family Mugilidae). WM pH was followed during PM storage at the optimum PM storage temperature in rested and exercised mullet. The effect of fish size on PM metabolism was also assessed in rested mullet. To further characterise the biochemical and metabolic changes occurring in the rested PM WM key metabolites (lactate, ATP, P_i, creatine and glycogen) were measured. Preservation of WM energy stores during harvesting is essential to allow the tissue to remain viable for as long as possible during PM storage. Buffering capacity of the rested WM and mean standard oxygen consumption in rested and exercised mullet were also measured and their significance to PM metabolism is discussed.

3.3 MATERIALS AND METHODS

3.3.1 Experimental timing and acclimation temperatures

Yellow-eye mullet captured in December 1996 and December 1998 were used in the experiments outlined in Table 3.1.

Table 3.1. *Sampling time, fish age and acclimation temperature of the five sets of experiments carried out on yellow-eye mullet.*

Experiment	Sampling time	Fish age (years)	Acclimation temperature* (°C)
Rested vs Exercised	Rested: July 2001	2.5	9.9 ± 0.2 °C
	Exercised: Aug/Sept 2000	1.5	12.0 ± 0.1 °C (21 days)
Different body size (rested fish)	Sept 1998	1.5	11.5 ± 0.1 °C
	July 2001	2.5	9.9 ± 0.2 °C
PM metabolism (rested fish)	Dec 1998	2.0	18.7 ± 0.3 °C
Buffering capacity	March 2001	2.25	19.8 ± 0.1 °C
Oxygen consumption (rested and exercised)	June/July 2001	1.5	11.4 ± 0.1 °C (40 days)

Values are the mean ± SEM
* Mean acclimated temperature calculated over the 14 days prior to sampling unless otherwise stated

3.3.2 Fish capture protocol

Rested harvesting

All yellow-eye mullet used in the experiments (excluding the exercised treatment) were harvested using rested harvesting techniques. Tank-rested yellow-eye mullet were anaesthetised with AQUI-S™ Plus at a concentration of 30.0 ± 0.5 mg/L (see Chapter 2: Harvesting Method; the appropriate concentration for anaesthetisation with AQUI-S™ Plus was determined in previous investigations). The fish were sedated after 5 min and were not suitable for handling until ~25 min. At this point 5 fish were transferred into a 20 L container with 10 L of seawater, containing the appropriate concentration of anaesthetic, and taken to the laboratory. Once in the anaesthetised state (insensitive to

cardiac puncture, ~50 min exposure), experimentation could begin. Experimental timing was recorded from the introduction of the anaesthetic and individual measurements were timed to the nearest minute.

Pre-harvest exercise

Yellow-eye mullet sampled in August and September 2000 were exercised prior to harvesting. This was carried out by draining the rearing tank down to a level of 30 cm and then 4 fish were individually dip-netted out of the tank (using a dip net strung with knotless mesh) and were forced to "flap" in the net (out of water) for a period of 1 min. Each fish was then transferred to a 50 L bin containing seawater and 30 ± 0.5 mg/L of AQUI-S^{TM} Plus.

This experiment was carried out on two different days to increase the number of replicates to 8. The ambient water temperature on the two days of experimentation was 12.0 ± 0.1 °C and 12.8 ± 0.1 °C. Experimental timing was recorded from when the fish were dip-netted into the anaesthetic. Fish were fully anaesthetised (State IV) after 10 min on both days the experiments were carried out.

Once fish were anaesthetised (rested and exercised mullet) a mixed venous blood sample was taken and the pH measured along with lactate and glucose (see Chapter 2: "Blood sampling and pH measurement").

3.3.3 Rested and exercised white muscle

Postmortem storage of white muscle

After blood sampling the fish were pithed (spinal cord ablation) using a traditional Japanese iki-jime tool. The fillets were excised from the carcass and then the right-hand-side fillets of the rested mullet and the left-hand-side fillets of the exercised mullet were placed on separate racks. The other fillets from the fish were used in another experiment in the laboratory) The racks of fillets were each placed in a chamber with inlet and outlet pipes for oxygen. The chamber, containing the fillets, was then submerged horizontally in a water bath at 5.0 ± 0.1 °C for the rested winter (2001) fish, 6.0 ± 0.1 °C for rested winter (1998) fish, 6.0 ± 0.1 °C and 6.4 ± 0.1 °C for the exercised winter fish and 10.0 ± 0.1 °C for the summer rested fish (refer to following section

"Choice of storage temperature"). The filter housing was flushed with humidified pure oxygen for ~5 min and then the oxygen flow was set to 50 mL/min ± 1.25%.

The flow-rate through the storage chambers was chosen as 50 mL/min as preliminary investigations using flow-rates of both 50 and 500 mL/min did not result in any significant difference in WM acidification rates.

Choice of storage temperature

A study carried out by Law & Jerrett (1996, unpublished results) showed that when rested yellow-eye mullet were stored at a temperature close to half their acclimated temperature in both summer and winter acclimated fish the decrease in cut-surface pH and accumulation of lactic acid in the WM was minimised after 20 h storage (Fig. 3.1, reproduced by permission of A.R. Jerrett and C&FR). Storage at half the acclimated temperature allowed better resolution of the PM changes occurring in the WM. On the basis of these results it was decided that the storage temperatures used in the current study would also be close to half the fishes ambient temperature (seawater temperature on the day of the rested winter fish sampling was 9.9 ± 0.1 °C), therefore the ischemic WM was stored at 5.0 ± 0.1 °C. The seawater temperature on the days of the exercised winter fish sampling experiment was 12.0 ± 0.1 °C and 12.8 ± 0.1 °C, therefore the ischemic WM was stored at 6.0 ± 0.1 °C and 6.4 ± 0.1 °C respectively. Seawater temperature on the day of the rested summer fish sampling was 20.1 ± 0.1 °C, therefore the ischemic WM was stored at 10.0 ± 0.1 °C.

White muscle pH measurement

The cut-surface WM pH measurements and WM samples (refer to Chapter 2: Materials and Methods) were taken from the WM of winter rested fish immediately after filleting and then ~12, 27, 37, and 50 h after anaesthetic had been introduced into the rearing tank. In winter exercised fish WM pH and samples were taken after filleting and then 6, 11, 16, 26 and 30 h after anaesthesia.

3.3.4 Different sized fish

Tank-rested yellow-eye mullet were sampled in 1998 and 2001 to determine if there was a difference in PM metabolism (as measured by WM pH) in different sized fish. Fish were anaesthetised, blood sampled, pithed, and then stored using the same protocol

as described for the rested fish. Ambient seawater temperature on the day of the rested September 1998 sampling was 12.0 ± 0.1 °C and in July 2000 was 9.9 ± 0.1 °C.

3.3.5 Characterisation of the post-mortem metabolism of rested white muscle

In December 1998 tank-rested yellow-eye mullet were sampled to further characterise the changes occurring in the WM during PM storage. Mullet were anaesthetised as described in Section 3.3.2 with fillets stored using the protocol set out in Section 3.3.3. The ambient seawater temperature on the day of the experiment was 20.8 ± 0.1 °C and therefore fillets were stored at 10.0 ± 0.1 °C (half the ambient temperature).

White muscle pH measurement and sampling

The cut-surface WM pH measurements and WM samples were taken from the fillets prior to going into storage and then 12, 27, 37, 50, and 57 h after introduction of the anaesthetic. WM samples were freeze-clamped and analysed for metabolites (lactate, P_i, ATP, creatine and glycogen) as described in Chapter 2.

3.3.6 Measurement of white muscle buffering capacity

The buffering capacity is defined as the amount of base (NaOH) required to titrate the pH of one gram of tissue (wet weight) by one pH unit. Buffering capacity carries the unit of β which is termed a 'slyke' (Castellini & Somero 1981). The in vitro buffering capacity of mullet WM due to non-bicarbonate compounds was assayed following the methods of Castellini & Somero (1981). Half a gram of mullet WM was freeze clamped (as for WM sampling) and ground to a powder in a liquid nitrogen cooled mortar and pestle. The powdered WM was then homogenised in 10.0 ± 0.1 mL of saline (0.9% NaCl). Sodium hydroxide (0.2 mol/L) was used to titrate the homogenate between pH values of approximately 6.5 and 7.5 (physiological pH range). The homogenate was stirred throughout the titration. Changes in pH were monitored using a pH meter (Model: PHM92; Radiometer, Copenhagen, Denmark) and a combination pH electrode (Model: PHC 2005-7; Radiometer, Copenhagen, Denmark). A micro-pipetter (Model: 10–100 µL; Eppendorf, Hamburg, Germany) was used to add 25 µL aliquots (5 µmoles) of NaOH to the homogenate.

3.3.7 Measurement of the metabolic rate of rested and exercised, anaesthetised mullet

Mullet sampled during June and July 2000 were either anaesthetised in the rearing tank using AQUI-S^{TM} Plus at a concentration of 30.0 ± 0.5 mg/L (rested fish) as described above (Section 3.3.1), or were dip-netted out of the tank and forced to flap in net (out of water) for ~1 min (exercised fish) and then placed in a plastic bin containing 10 L of seawater and 30 ± 0.5 mg/L AQUI-S^{TM} Plus (see Section 3.3.2). Only one fish could be sampled at a time. A total of 5 rested and 2 pre-harvest exercised fish were measured. Unfortunately it was not possible to carry out further experiments on pre-harvest exercised fish to increase the number of replicates.

Rested fish were anaesthetised and able to be handled after ~25 min and exercised fish had reached the same stage of anaesthesia after only 10 min. Once anaesthetised each fish was transferred to a Perspex respirometer box (60 x 290 x 100 mm) in the laboratory. Seawater was introduced to the respirometer via a plenum at one end and exited via a second plenum at the other end in a flow-through manner. This arrangement ensured that the water delivered to the fish was bubble free and that the chamber was held at constant depth. Influent water entered the chamber through a short length (17 mm) of soft rubber hose (9 mm dia.). This tube was inserted into the mouth of the fish ~10 mm to irrigate the gills.

Fish were kept under anaesthesia (Stage IV to Stage V) in the respirometer by maintaining the anaesthetic concentration of the irrigating seawater solution at 25 mg/L. This was achieved by providing a stirred reservoir of anaesthetic stock solution at the appropriate concentration. This solution was delivered to a stirred seawater/anaesthetic mixing chamber (Model: W10PR Filterpure Housing, Microlene, U.S.A) at a rate of 0.69 ± 0.003 mL/min (± SEM) via a peristaltic pump (Model: 7521-4S Masterflex Console Drive, Cole-Palmer Instrument Co., Vernon Hills, IL, USA; pump head 70 13-20; C-Flex tubing L/S 13). The flow rate of the seawater was controlled by a flow-meter (Model: A10HS-PC; Platon Instrumentation, Basingstoke, England) with the flow set at 1.5 L/min. The irrigating seawater flow and the anaesthetic stock solution flow rates were confirmed by repeated weight/time measurements.

Temperature of the irrigating seawater was monitored with a Type T thermocouple temperature probe (Model: 219-4674; RS Components, Auckland, New Zealand) in the influent plenum (the difference between the inflow and outflow water was negligible). The temperature was not controlled, being at the ambient seawater temperature at which the fish were held prior to anaesthesia.

Measurement of dissolved oxygen in the influent and effluent water

The oxygen consumption rate was monitored using a dissolved oxygen flow-through probe (Model: MI-730 Microelectrodes, Inc., Bedford, NH, USA). Water from either the influent or effluent flow was diverted through the electrode for measurement by using a pneumatic valve. This was set on a cycling time switch so that the oxygen level in the effluent water was measured for 10 min and the influent water for 5 min. The probe was calibrated in situ. The influent water was delivered from a 700 L elevated, aerated, constant head reservoir. The water was oxygen saturated, with this being confirmed by measurements (Model: 550 DO meter, Yellow Springs Instruments Incorporated, Yellow Springs, OH, USA). A second reservoir of seawater was sparged with oxygen free nitrogen gas to act as the zero oxygen standard.

Measurement recording

Seawater temperature and dissolved oxygen were recorded using a chart recorder (Model: LR4100E, Yokogawa Electric Corporation, Tokyo, Japan).

Calculation of respiratory variables

Standard oxygen consumption ($\dot{M}O_2$) was calculated by the Fick principle:

$$\dot{M}O_2 = \frac{(PO_2 \text{ inlet} - PO_2 \text{ outlet}) \times Q_w \times \alpha wO_2}{\text{body weight}}$$

where Q_w is the constant water flow through the respirometer (L/min) and αwO_2 is the O_2 solubility in the seawater (μmol/L/torr) (as described by Maxime et al. 2000). As the oxygen electrode measured the change in oxygen in percent, this was converted to PO_2 (partial pressure of oxygen) using the barometric pressure and converting the measurement to torr.

3.3.8 Statistical analysis

All times, pH values and biochemical values stated in the text are the mean ± standard error of the mean (SEM). Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows Version 6.00 (SPSS Inc.) and Microsoft® Excel 2000.

3.4 RESULTS

3.4.1 Rested and exercised mullet

Post-harvest condition

The exercised group of fish were larger in weight than the rested fish ($P = 0.07$), but the fork length of the fish in the two groups was similar (Table 3.2). As a result the CF of the rested fish was significantly lower than the exercised fish. The HSI of the exercised fish was higher than the rested group, however, due to the large variation in liver weights it was not significantly different. The two groups of fish were from the same population, with the rested fish being a year older than the exercised group. Exercised fish became anaesthetised much faster than rested fish and consequently were sampled significantly earlier than rested fish (see Table 3.2). The blood pH of exercised fish was significantly lower than the rested fish and exercised fish had significantly higher blood lactate and glucose levels (Table 3.2).

Table 3.2. *Size, condition and whole blood measurements from winter rested and exercised mullet.*

	Rested 2001 (n = 5)	**Exercised 2000 (n = 8)**
Weight (g)	259.7 ± 32.2	375.7 ± 48.2
Length (mm)	271 ± 10	288 ± 14
Sex	3M; 2F	4M; 4F
CF[1]	1.28 ± 0.05	1.52 ± 0.04*
HSI[2]	2.95 ± 0.29	4.22 ± 0.72
Pithing time (h)	0.83 ± 0.04	0.62 ± 0.05*
Blood pH	7.82 ± 0.06	7.27 ± 0.05*
Blood lactate (mmol/L)	1.8 ± 0.7	10.7 ± 1.5*
Blood glucose (mmol/L)	5.5 ± 0.4	8.6 ± 0.6*

[1]Condition factor (CF) = weight (g)/length (mm^3) x 100000 (Love 1980).
[2]Hepatosomatic index (HSI) = liver weight (g)/weight (g) x 100 (Love 1980).
Values are the mean ± SEM, n = 5.
* Significantly different from the rested 2001 fish ($P < 0.005$; Student's t-test).

The relationship between mixed venous blood pH and blood lactate concentration is shown in Fig. 3.2. As blood lactate concentration increased (i.e. as a result of exercise) the blood pH was progressively acidified. There was a similar correlation between mixed venous blood pH and blood glucose concentration (Fig. 3.3). Although the relationship was not as strong as that seen with lactate, the blood became acidified while the blood glucose increased.

Post-mortem white muscle pH profiles

The PM cut-surface pH profiles of the WM for rested and exercised mullet are shown in Fig. 3.4. The initial WM pH in the exercised WM was ~0.5 units lower than in rested WM. There was also increased variation in the measured pH values in the exercised fish. During PM storage the initial rate at which the WM cut-surface pH decreased was similar in both rested and exercised fish (Fig. 3.4). However, when the WM pH values were converted into $[H^+]$ it was clear that the rate of acidification in the WM of exercised mullet was much faster than in rested mullet (Fig. 3.5). The exercised WM had reached its ultimate pH after ~16 h storage, whereas the rested WM did not reach its ultimate pH until after 25 h storage. The ultimate pH of the rested WM was ~0.1 pH units higher than the exercised treatment.

3.4.2 Winter acclimated rested mullet: different sized fish

The mullet sampled in winter 2001 were larger in weight and length, but had lower CF and HSI (Table 3.3). Mullet sampled in winter 1998 had slightly higher blood lactate levels reflected in the lower blood pH. The relationship between blood pH and blood lactate concentration is shown in Fig. 3.6. There was a similar relationship between blood pH and blood lactate as seen in rested and exercised mullet. There was no difference in blood glucose levels between the two groups of fish. The PM cut-surface pH of the WM from the two different populations of winter acclimated rested mullet is shown in Fig. 3.7. Mullet sampled from different populations that were of different size did not show any difference in cut-surface pH profile during PM storage.

Table 3.3. *Size, condition and whole blood measurements from rested winter acclimated mullet.*

	Winter 2001 (n = 5)	**Winter 1998 (n = 5)**
Weight (g)	259.7 ± 32.2	176.5 ± 20.0
Length (mm)	271 ± 10	228 ± 9*
CF[1]	1.28 ± 0.05	1.46 ± 0.01*
HSI[2]	2.95 ± 0.29	4.26 ± 0.34*
Pithing time (h)	0.83 ± 0.04	0.95 ± 0.04
Blood pH	7.82 ± 0.06	7.67 ± 0.08
Blood lactate (mmol/L)	1.8 ± 0.7	3.9 ± 0.9
Blood glucose (mmol/L)	5.5 ± 0.4	6.7 ± 0.8

[1]Condition factor (CF) = weight (g)/length (mm^3) x 100000 (Love 1980).
[2]Hepatosomatic index (HSI) = liver weight (g)/weight (g) x 100 (Love 1980).
Values are the mean ± SEM, n = 5.
* Significantly different from the winter 2001 fish ($P < 0.05$; Student's t-test).

3.4.3 Characterisation of the post-mortem metabolism of rested white muscle

Post-harvest condition

The mean weight of the 5 mullet sampled in December 1998 was 214.8 ± 23.3 g and mean fork length was 247 ± 7 mm. The CF of the mullet was 1.40 ± 0.04 and HSI was 2.93 ± 0.41. Blood was sampled from the rested fish at a mean time of 0.61 ± 0.04 h after introduction of the anaesthetic. The mean blood pH was 7.69 ± 0.04, blood lactate 1.9 ± 0.6 mmol/L and blood glucose 6.1 ± 0.3 mmol/L. The immediate post-harvest, pre-storage cut-surface pH of the D1 WM in the rested fish was measured at a mean time of 1.13 ± 0.12 h and was 7.61 ± 0.02. The mean immediate post-harvest metabolite levels were as follows: lactate = 12.7 ± 1.0 μmol/g; ATP = 7.4 ± 0.7 μmol/g; P_i = 22.9 ± 2.1 μmol/g; creatine = 12.6 ± 1.0 μmol/g; glycogen = 37.6 ± 5.6 μmol/g.

Post-mortem white muscle pH profiles

The PM pH profiles of rested WM sampled in December 1998 is shown in Fig. 3.8. The WM pH dropped rapidly from 0 to 27 h and had reached the ultimate pH of ~6.40 27 h post-anaesthesia.

Post-mortem white muscle metabolite profiles

In rested mullet [lactate] increased rapidly in the WM over the first 27 h of storage (Fig. 3.9a). After 27 h the [lactate] reached a peak of ~70 μmol/g muscle mass, and did not change over the remaining storage period. There was a strong linear relationship (r^2 = 0.93) between WM pH and WM [lactate] (Fig. 3.10a) over the range that was measured (pH ~7.7 to ~6.1). The cut-surface pH measurement approximated the WM lactate concentration very well. Converting the WM pH into [H^+] showed that there was an exponential relationship between H^+ and lactate concentration in the WM (Fig. 3.10b).

There was rapid depletion of ATP in the WM during PM storage with levels reaching <1 μmol/g muscle mass after 27 h storage. The relationship between pH and ATP is shown in Fig 3.11a. The pH/lactate relationship was slightly stronger than the pH/ATP relationship demonstrated by the lower r^2 values for ATP (see Figs. 3.10a and 3.11a legends). Even so it shows that cut-surface pH is a fast and accurate way to

describe the physiological state of the WM. When the WM pH was converted to $[H^+]$ there was a distinct biphasic relationship with WM ATP concentration (Fig. 3.10b). While ATP was still present in the WM (greater than ~3 μmol/g muscle mass) there was minimal acidification of the WM. When ATP levels were <2 μmol/g muscle mass $[H^+]$ was high.

The negative linear relationship between WM lactate and ATP concentration is shown in Fig. 3.12. At high ATP concentrations (start of PM storage) lactate levels were low and conversely, as ATP was depleted lactate levels rose.

There was a ~12 h delay in accumulation of P_i in the rested WM during PM storage (Fig. 3.9c). After 12 h storage $[P_i]$ rose steadily and peaked at ~40-45 μmol/g muscle mass after 37 h. Figure 3.13a shows the relationship between WM pH and P_i concentration. In general as WM $[P_i]$ increased pH decreased, however, once the WM had reached its ultimate pH (~6.4) $[P_i]$ continued to increase (see also Figs 3.8 & 3.9c). On conversion of the WM pH to $[H^+]$ there appeared to be a step change in acidification occurring between ~30 to 35 μmol/g muscle mass (Fig. 3.13b). Below this level $[H^+]$ was negligible, and higher than this the majority of $[H^+]$ measurements were between ~3 and 4.5 x 10^{-7} mol/L. The relationship between WM P_i and ATP concentration is shown in Fig. 3.14. While ATP levels in the WM were high $[P_i]$ was low. As ATP became depleted (<1 μmol/g) $[P_i]$ rose and was variable in a similar pattern as WM pH and $[P_i]$.

Accumulation of creatine in mullet WM followed a similar pattern to that seen with lactate i.e. a rapid rise to a maximum (~22-24 μmol/g muscle mass) after 27 h. Although many of the creatine measurements were higher than 20 μmol/g muscle mass and cannot be reliably assessed, the initial rested values were below this level and were therefore accurate. Consequently, only the overall trend of creatine accumulation will be discussed.

Mullet WM glycogen levels (expressed as glucosyl units) are shown in Fig. 3.9e. Prior to storage the rested WM contained ~35-40 μmol/g muscle mass of glycogen. The concentration then steadily dropped to be significantly different from starting values at 37 h. After 37 h storage [glycogen] remained at ~15 μmol/g muscle mass.

3.4.4 White muscle buffering capacity

The buffering capacity of mullet WM acclimated to summer temperatures was measured by titrating the muscle homogenate with NaOH (see Fig. 3.15). WM from a group of 5 rested mullet was used to measure the buffering capacity with the mean being 78.7 ± 3.6 Slykes (μmoles of base required to titrate the pH of one gram wet weight of muscle by one pH unit).

3.4.5 Resting and post-exercise oxygen consumption

A typical chart-recorder trace from the measurement of O_2 consumption for a rested winter mullet is shown in Fig. 3.16. The mean standard oxygen consumption ($\dot{M}O_2$) for the rested fish was calculated when the oxygen content of the out-flow water had stabilised (~1 h). The $\dot{M}O_2$ of 5 rested fish sampled in winter was 26.1 ± 2.5 μmol/min/kg body mass (mean weight = 443.0 ± 98.9 g, ranging from 211.0 g to 730.3 g). The mean $\dot{M}O_2$ of the 2 fish forced to exercise was measured as soon as the fish were anaesthetised (~10 min) rather than allowing the oxygen content of the outflow water to stabilise as in the resting measurement). The average $\dot{M}O2$ of the exercised fish was 75.0 μmol/min/kg body mass (average weight = 84.7 g). Assuming that 60% of the body mass was WM (Moyes & West 1995) then the rate of oxygen consumption in the exercised WM can be estimated as 45 μmol/min/kg muscle mass (0.045 μmol/min/g muscle mass). This rate was not calculated in the rested WM because at rest it was assumed that the oxygen consumption of the WM would be minimal compared with the rest of the body.

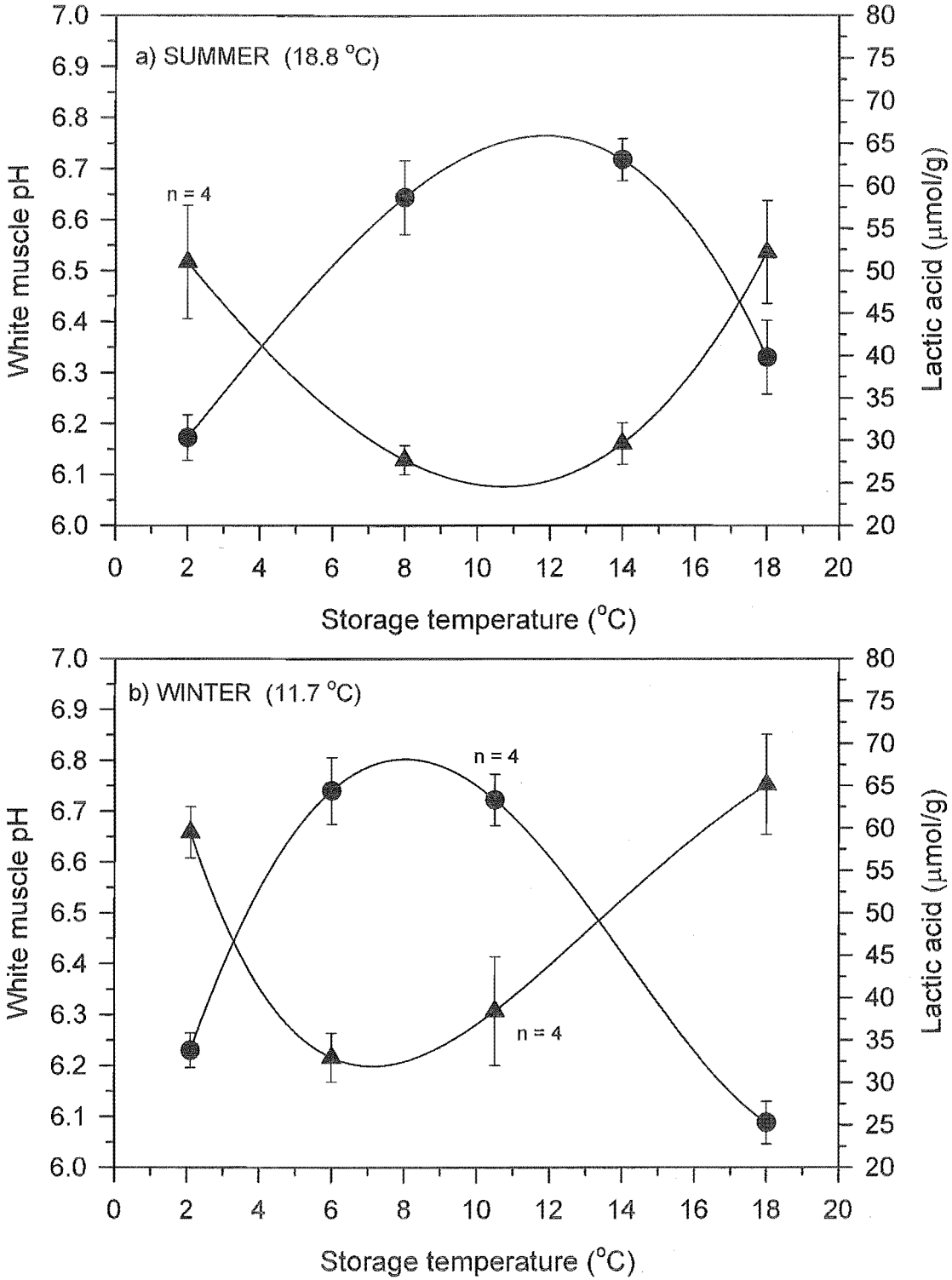

Figure 3.1: *Post-mortem cut-surface pH (●) and [lactate] (▲) of the epaxial white muscle of rested yellow-eye mullet acclimated to a) summer temperatures and b) winter temperatures, measured at 21.6 h and 21.0 h (± 0.1 h SEM) respectively, after anaesthesia in contrast to temperature of 35‰ seawater storage medium. Values are the mean ± SEM, n = 5 unless otherwise stated. Reproduced by permission of A.R. Jerrett and C&FR.*

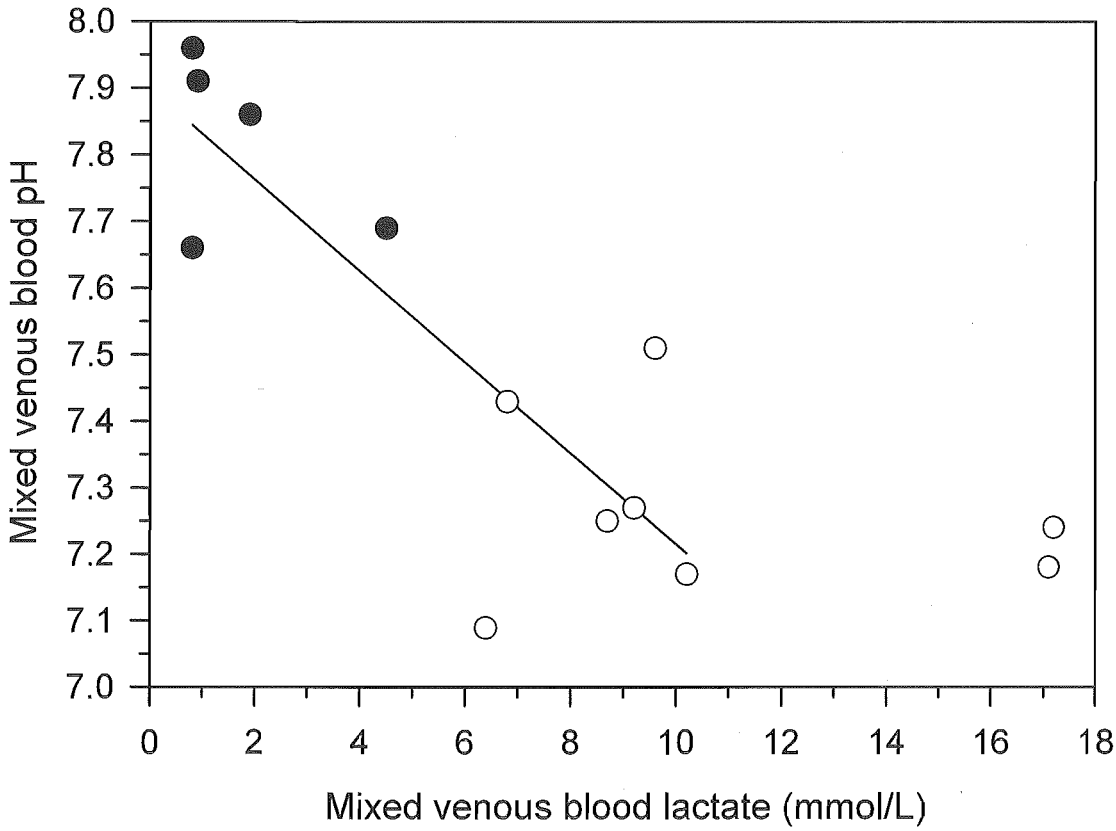

Figure 3.2: *Relationship between mixed venous blood pH and blood lactate concentration in rested (winter 2001 ●) and exercised (winter 2000 ○) yellow-eye mullet. The relationship is described by the linear regression equation: y = -0.068x + 7.90; r^2 = 0.70. The regression equation was calculated excluding the two high blood lactate concentrations at the bottom right of the graph. The correlation coefficient for the relationship is –0.84.*

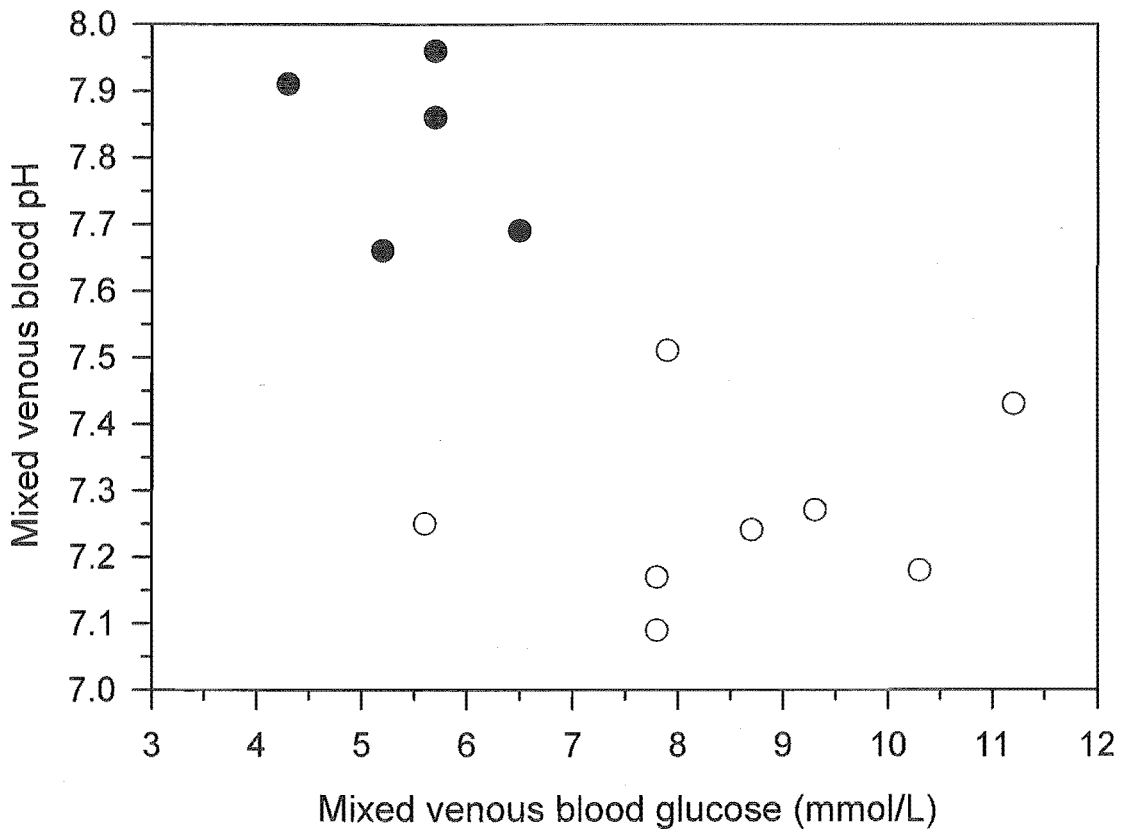

Figure 3.3: *Relationship between mixed venous blood pH and blood glucose concentration in rested (winter 2001 ●) and exercised (winter 2000 ○) yellow-eye mullet. The correlation coefficient for the relationship is –0.65.*

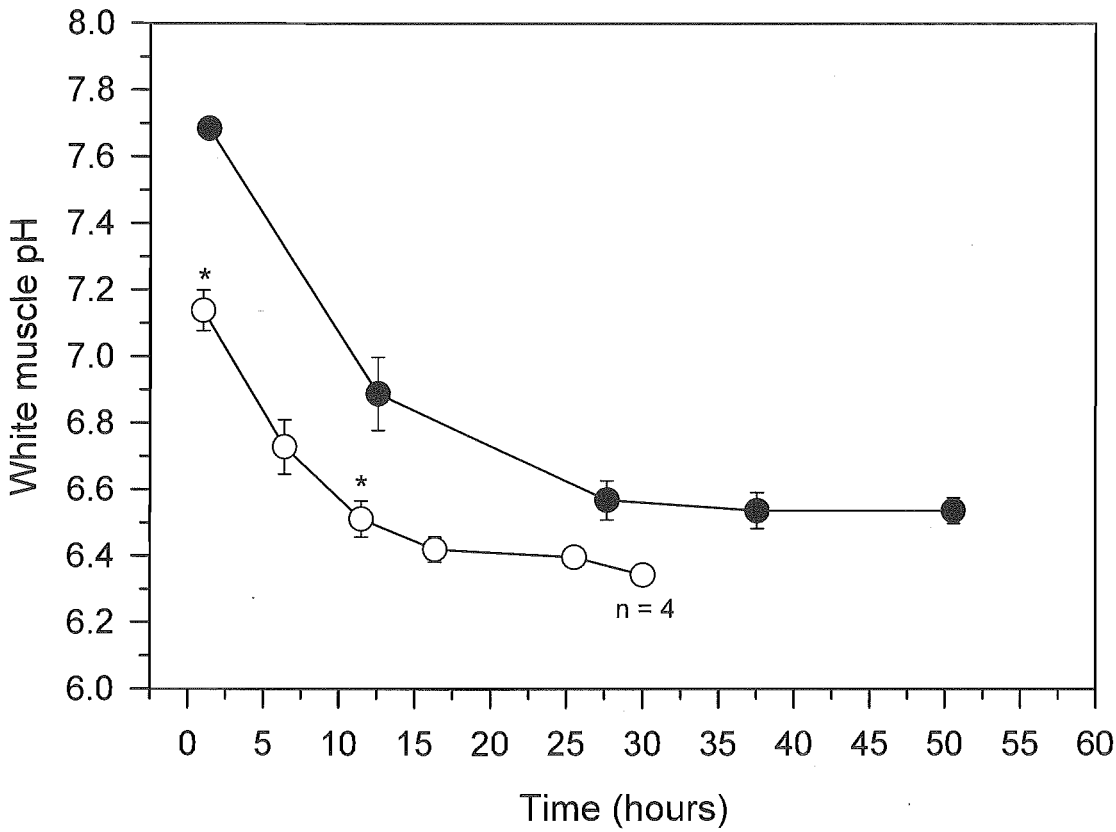

Figure 3.4: *Post-mortem cut-surface pH of the epaxial white muscle from rested (winter 2001 ●) and exercised (winter 2000 ○) yellow-eye mullet. Fillets were stored under normobaric conditions with oxygen flow 50 mL/min ± 1.25%. and at half the ambient temperature of the fish (5.0 ± 0.1 °C for winter rested; 6.0 and 6.4 ± 0.1 °C for winter exercised). Values are the mean ± SEM. For rested fish n = 5, for exercised fish n = 8 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the rested value at the corresponding sample time.*

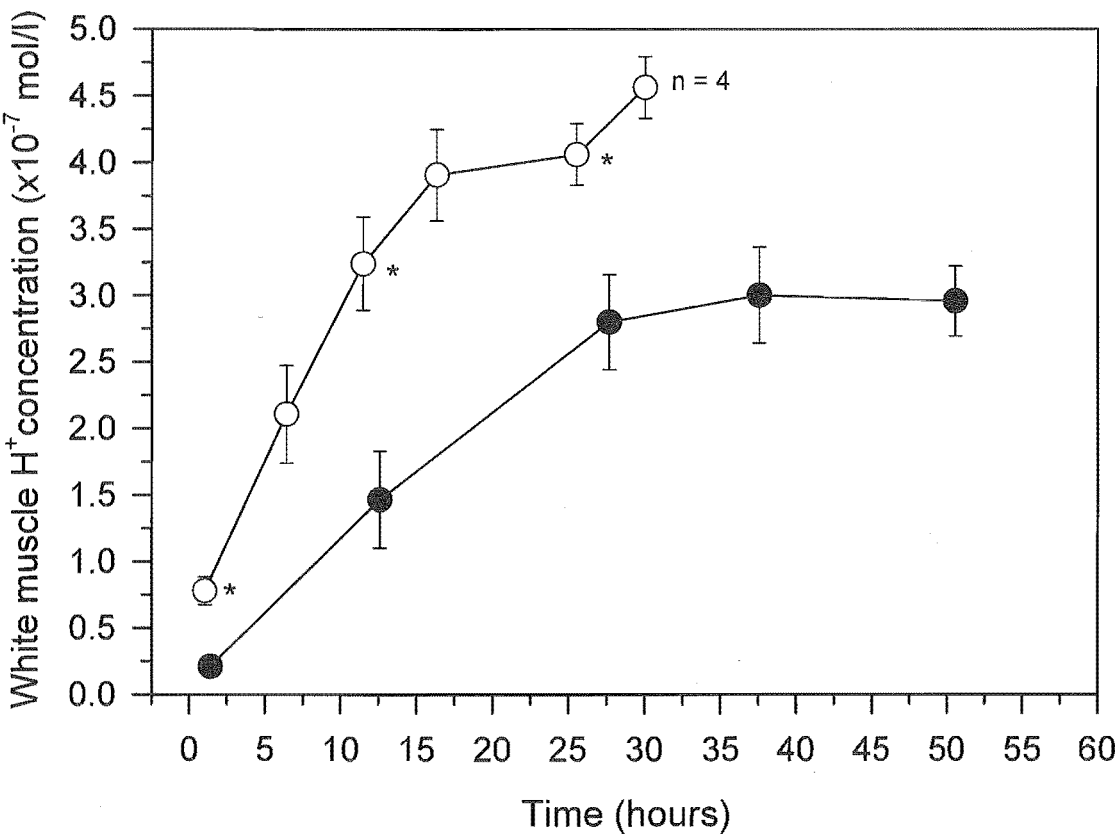

Figure 3.5: *Post-mortem H^+ concentration in the epaxial white muscle from rested (winter 2001 ●) and exercised (winter 2000 ○) yellow-eye mullet. See Fig. 3.4 legend for storage details. Values are the mean ± SEM. For rested fish n = 5, for exercised fish n = 8 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the rested value at the corresponding sample time.*

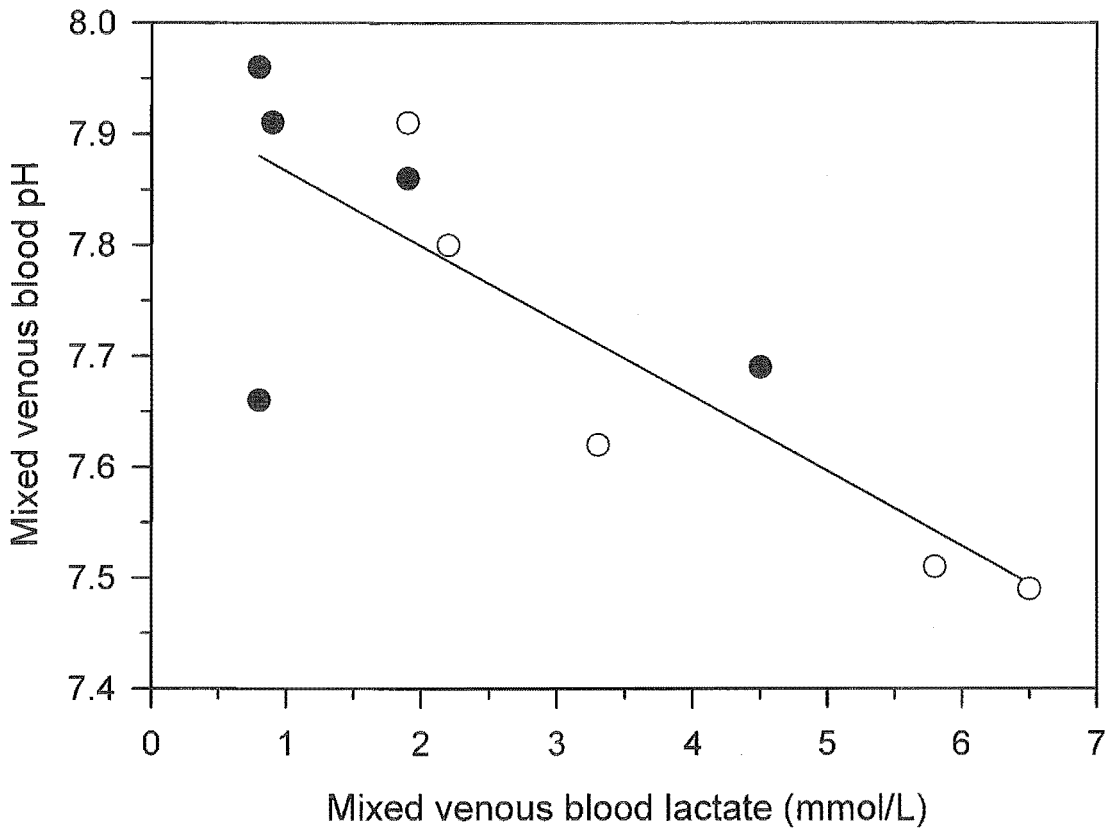

Figure 3.6: *Relationship between mixed venous blood pH and blood lactate concentration in rested winter 2001 (●) and rested winter 1998 yellow-eye mullet (○). The relationship is described by the linear regression equation: $y = -0.068x + 7.93$, $r^2 = 0.68$. The correlation coefficient for the relationship is –0.70.*

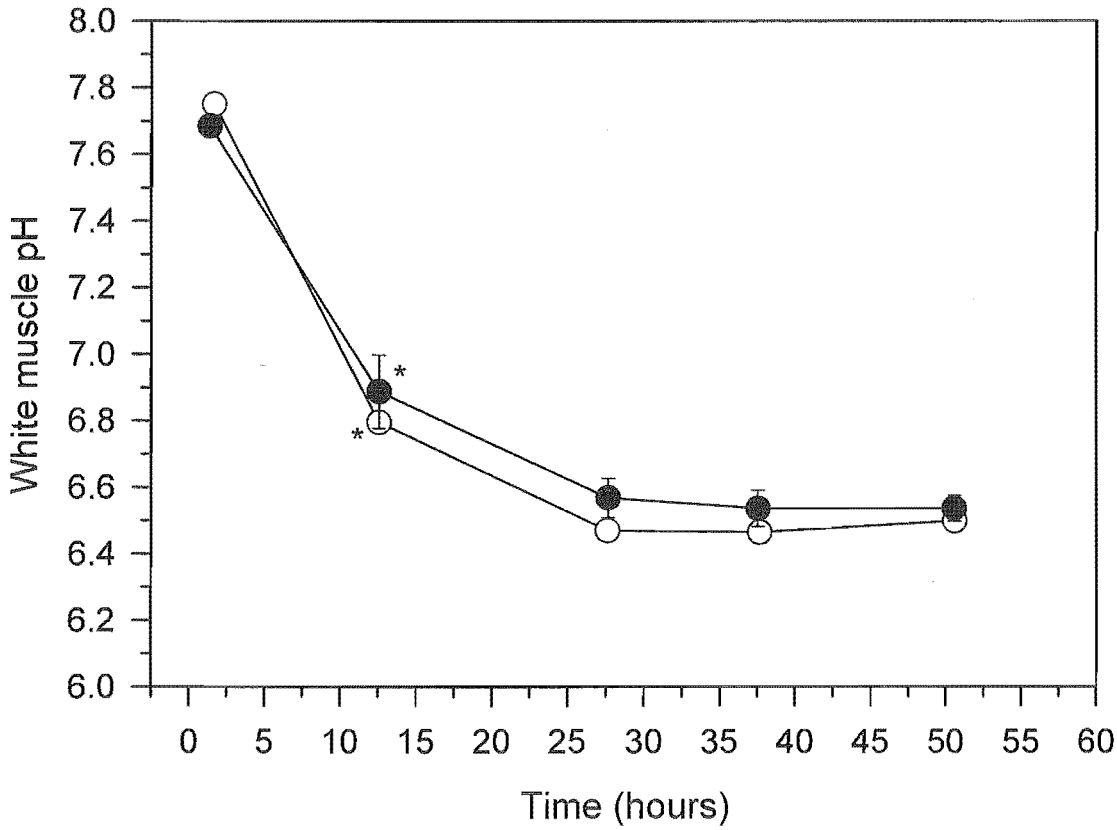

Figure 3.7: *Post-mortem cut-surface pH of the epaxial white muscle from winter 1998 rested (○) and winter 2001 rested (●) yellow-eye mullet. See Fig. 3.4 legend for storage details. Winter 1998 ambient temperature 12.1 ± 0.01 °C: storage temperature 6.0 ± 0.01 °C; winter 2001 ambient temperature 9.9 ± 0.01 °C: storage temperature 5.0 ± 0.01 °C). Values are the mean ± SEM, n = 5. * indicates a significant difference (Sign test; P < 0.05) from the initial pre-storage value – only first instance marked.*

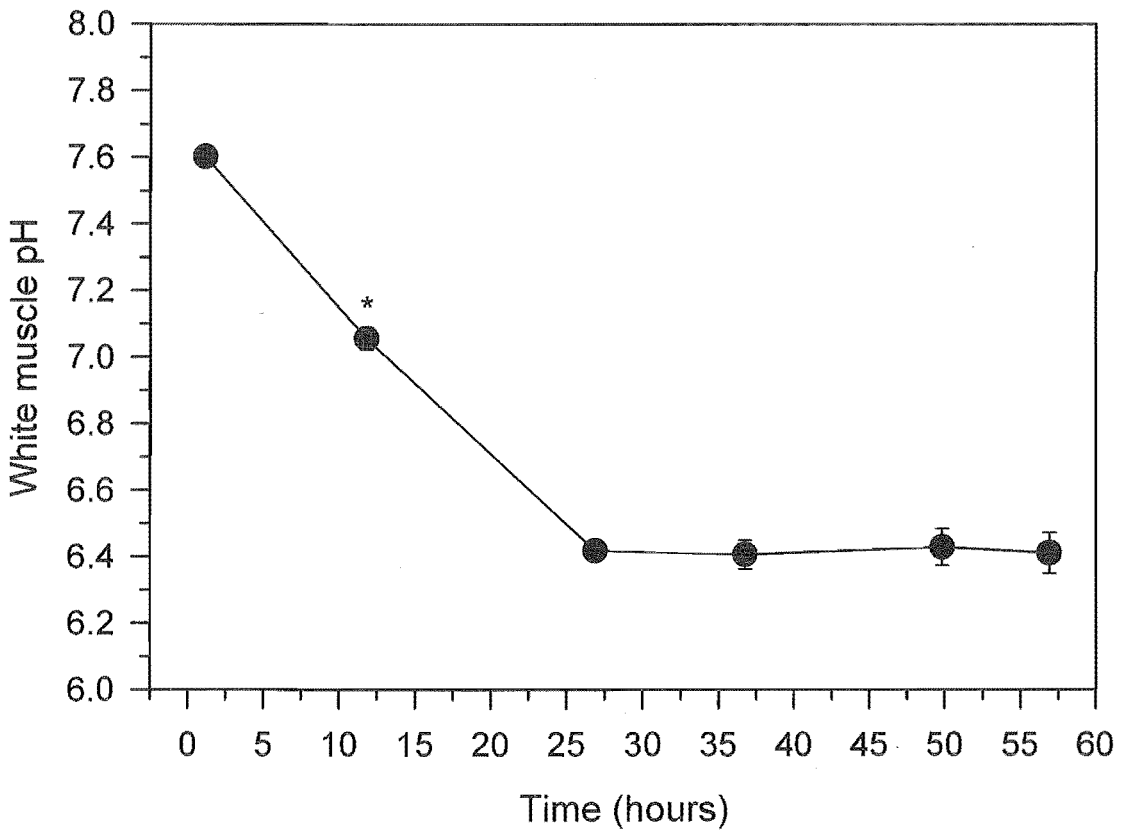

Figure 3.8: *Post-mortem cut-surface pH of the epaxial white muscle from summer (1998) rested yellow-eye mullet. See Fig. 3.4 legend for storage details. Summer 1998 ambient temperature 20.8 ± 0.01 °C: storage temperature 10.0 ± 0.01 °C. Values are the mean ± SEM, n = 5. * indicates a significant difference (Sign test; P < 0.05) from the initial pre-storage value – only first instance marked.*

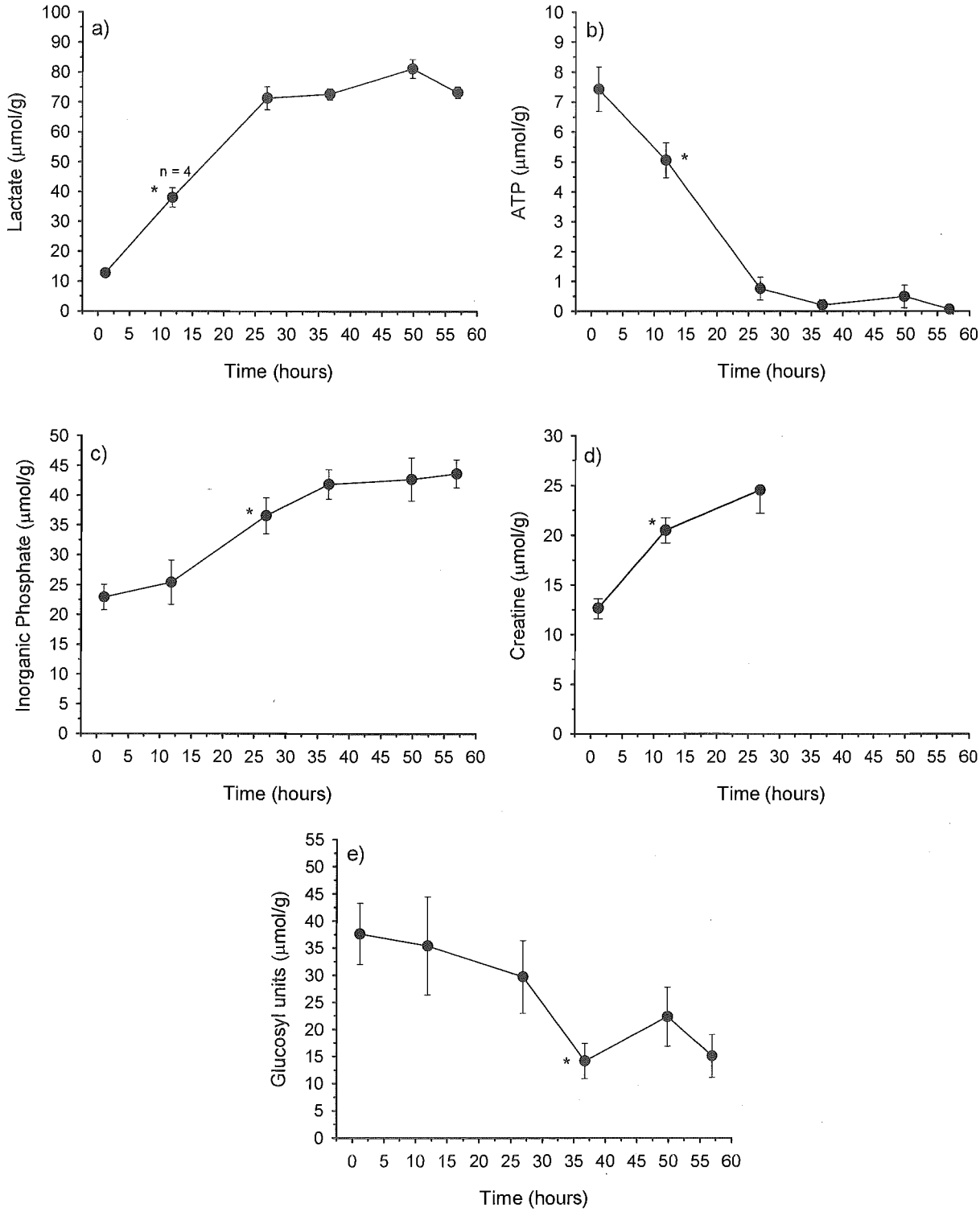

Figure 3.9: *Post-mortem changes in a) lactate, b) ATP, c) P_i, d) creatine and e) glycogen (glucosyl units) concentration in the epaxial white muscle of summer (1998) rested yellow-eye mullet. See Fig. 3.4 & 3.8 legend for storage details. Values are the mean ± SEM; n = 5. * indicates a significant difference (Sign test; $P < 0.05$) from the initial pre-storage value – only first instance marked.*

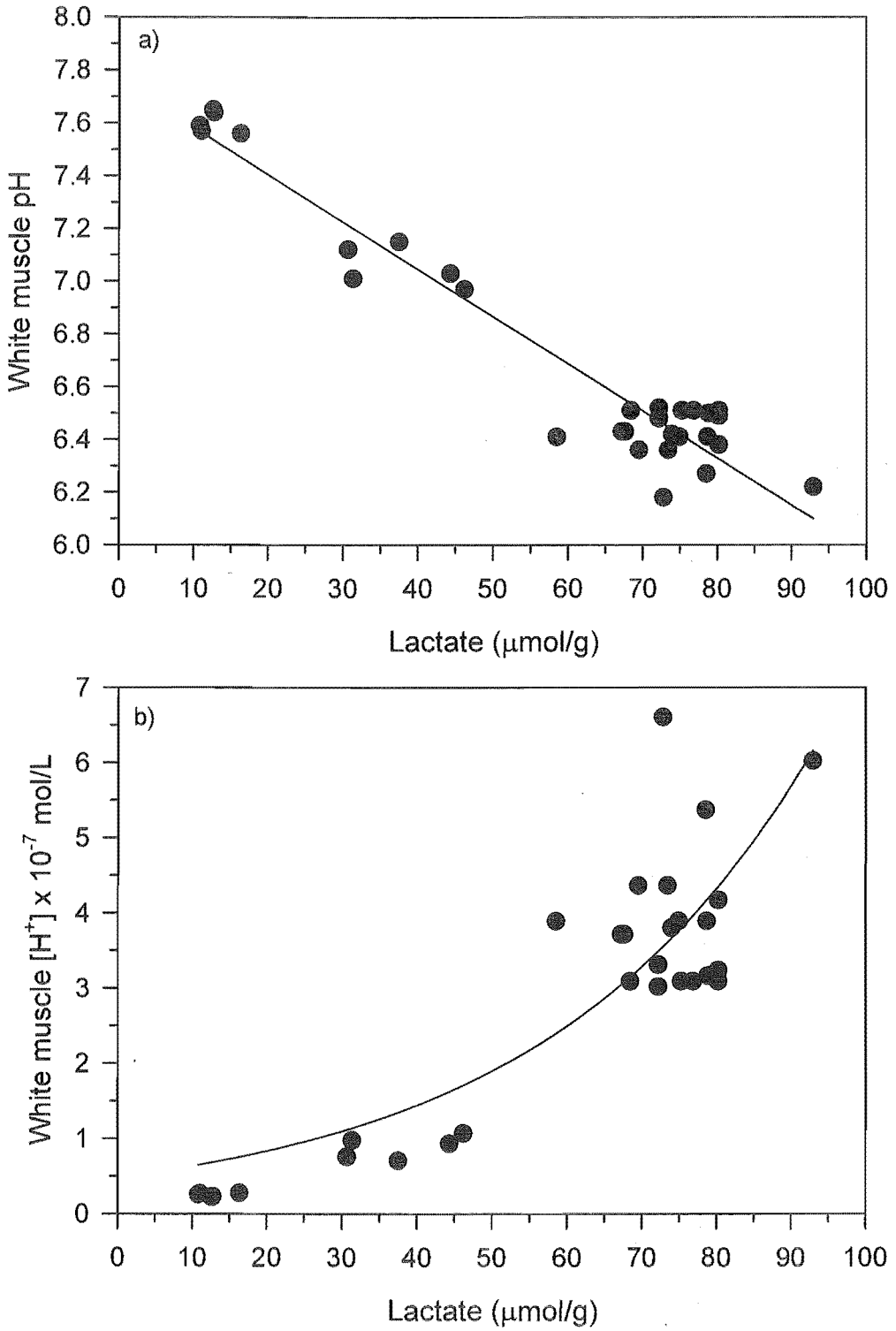

Figure 3.10: *Relationship between a) post-mortem cut-surface pH and [lactate] and b) post-mortem H^+ concentration and [lactate] in the epaxial white muscle from summer (1998) rested yellow-eye mullet. The pH/[lactate] relationship is described by the linear regression equation: $y = 0.017x + 7.72$, $r^2 = 0.93$. The correlation coefficient for the relationship is –0.96. The $[H^+]$/[lactate] relationship is described by the exponential equation $y = 0.48e^{0.028x}$, $r^2 = 0.75$. The correlation coefficient for the relationship is 0.88.*

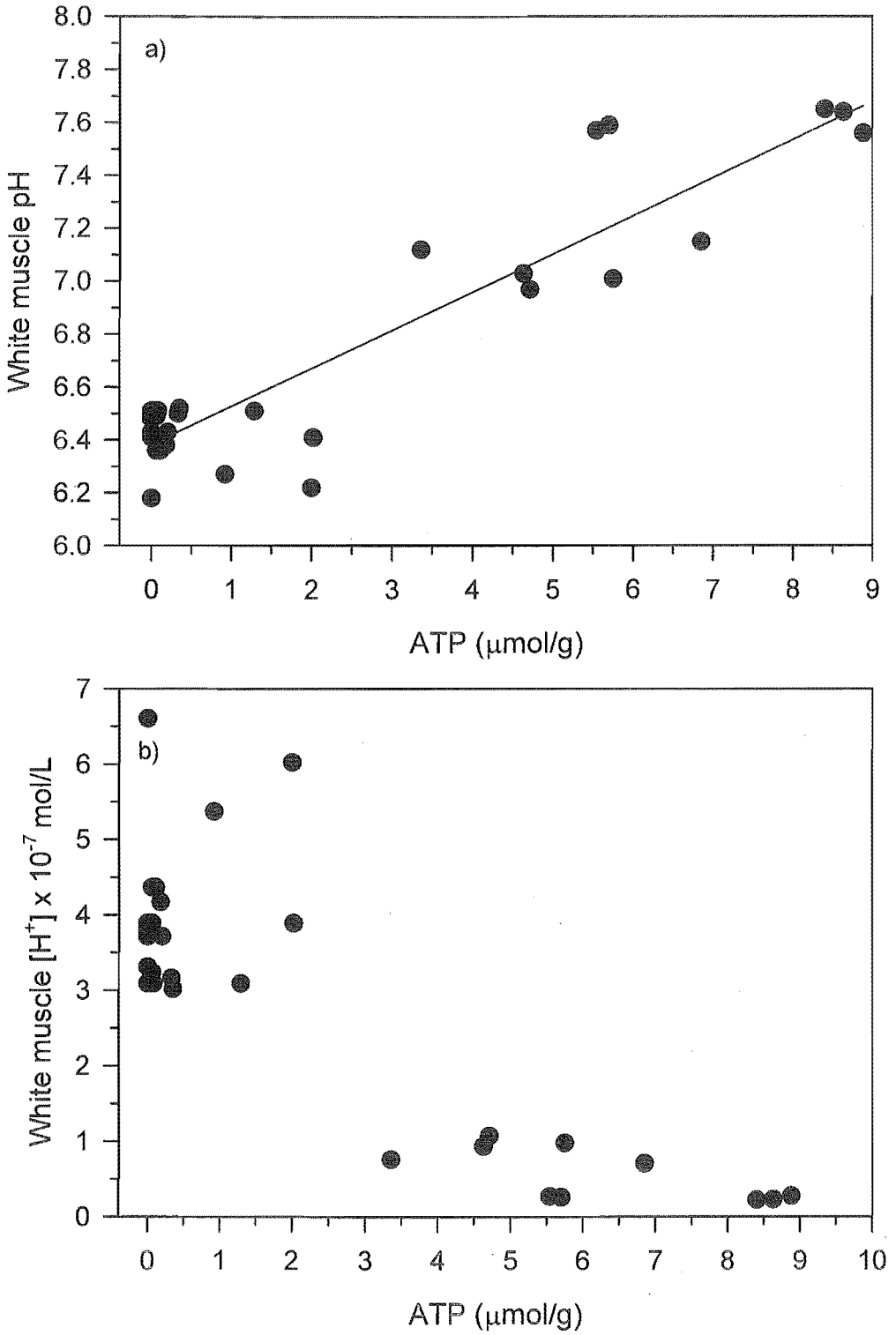

Figure 3.11: *Relationship between a) post-mortem cut-surface pH and [ATP] and b) post-mortem H^+ concentration and [ATP] in the epaxial white muscle from summer (1998) acclimated rested yellow-eye mullet. The pH/[ATP] relationship is described by the linear equation: $y = 0.144x + 6.38$, $r^2 = 0.86$ (solid line). The correlation coefficient for the pH/[ATP] relationship is 0.93, and for the $[H^+]$/[ATP] relationship – 0.82.*

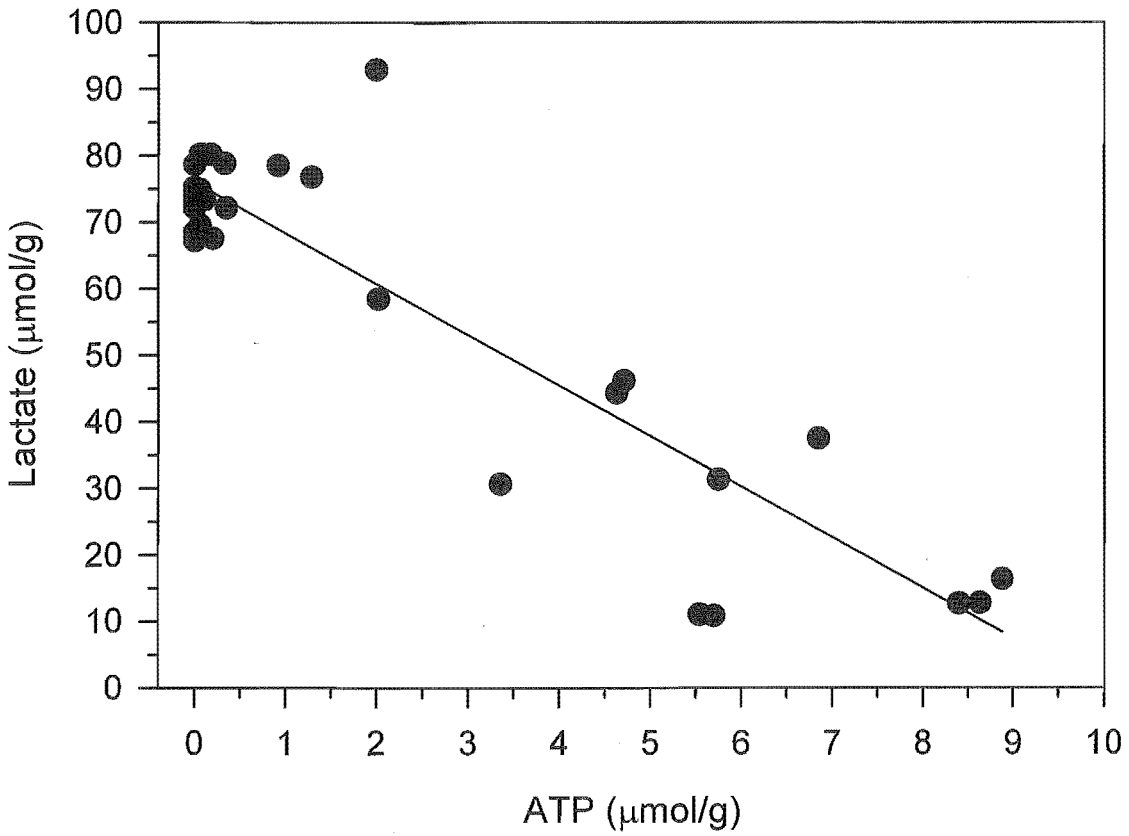

Figure 3.12: *Relationship between post-mortem white muscle [lactate] and [ATP] in the epaxial white muscle from summer (1998) acclimated rested yellow-eye mullet. The relationship is described by the linear equation:* $y = -7.94x + 76.21$, $r^2 = 0.83$ *(solid line). The correlation coefficient for the relationship is -0.90.*

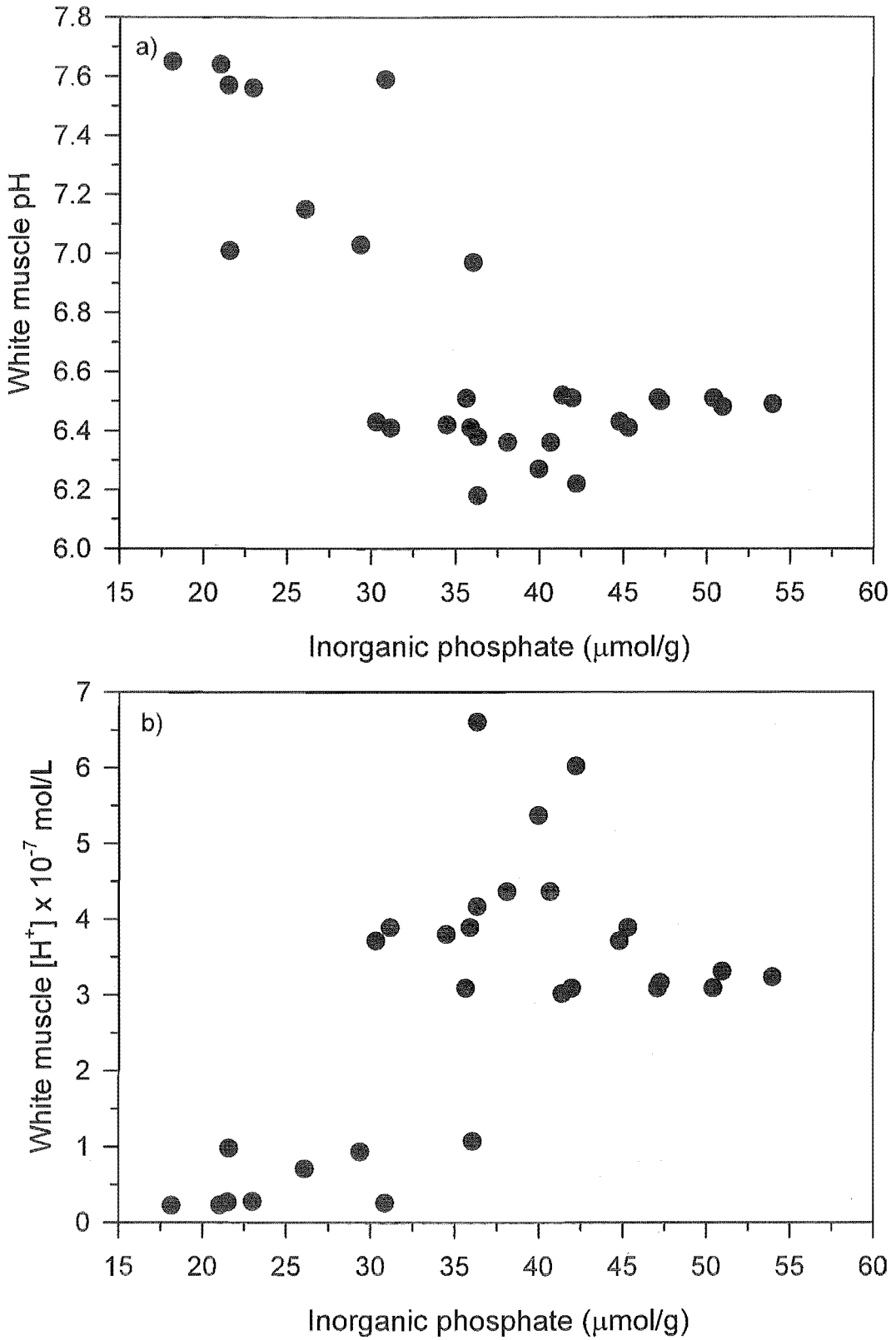

Figure 3.13: *Relationship between a) post-mortem cut-surface pH and [P_i] and b) post-mortem H^+ concentration and [P_i] in the epaxial white muscle from summer (1998) acclimated rested yellow-eye mullet. The correlation coefficient for the pH/[P_i] relationship is -0.73 and for the [H^+]/[P_i] relationship 0.61.*

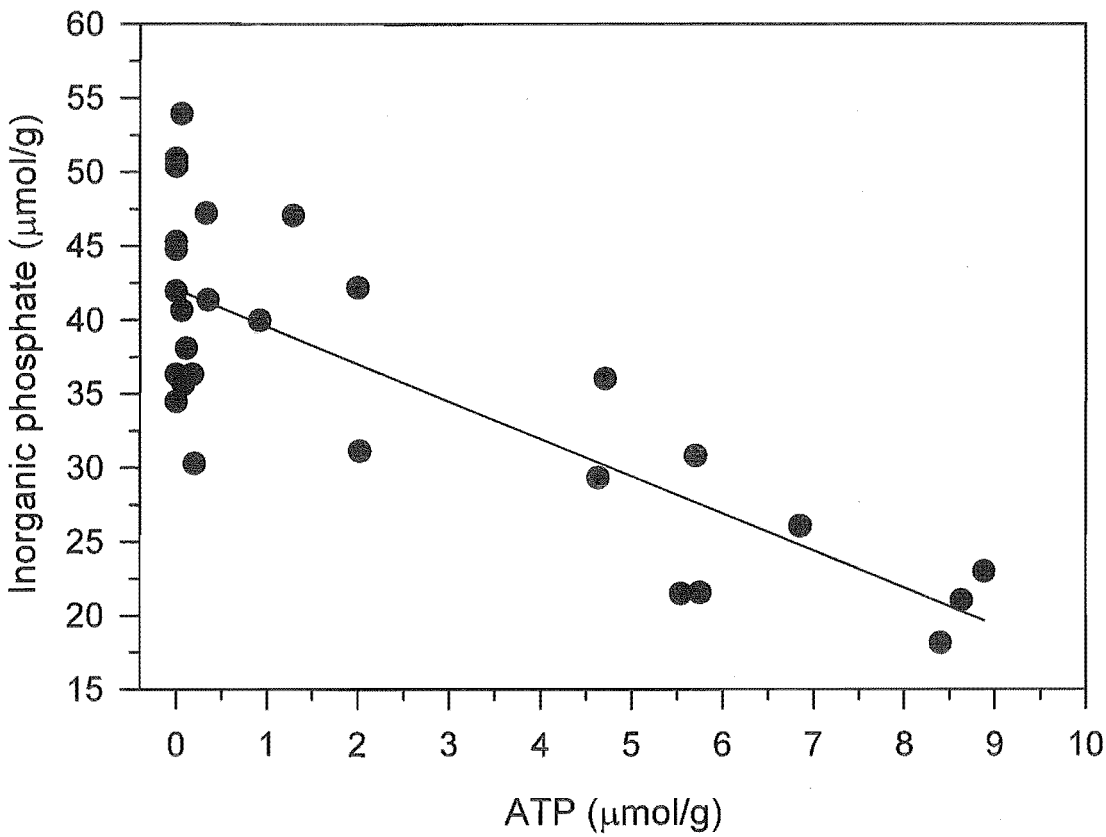

Figure 3.14: *Relationship between post-mortem white muscle [P_i] and [ATP] in the epaxial white muscle from summer (1998) acclimated rested yellow-eye mullet. The relationship is described by the linear equation: $y = -2.53x + 42.07$, $r^2 = 0.64$. The correlation coefficient for the relationship is -0.80.*

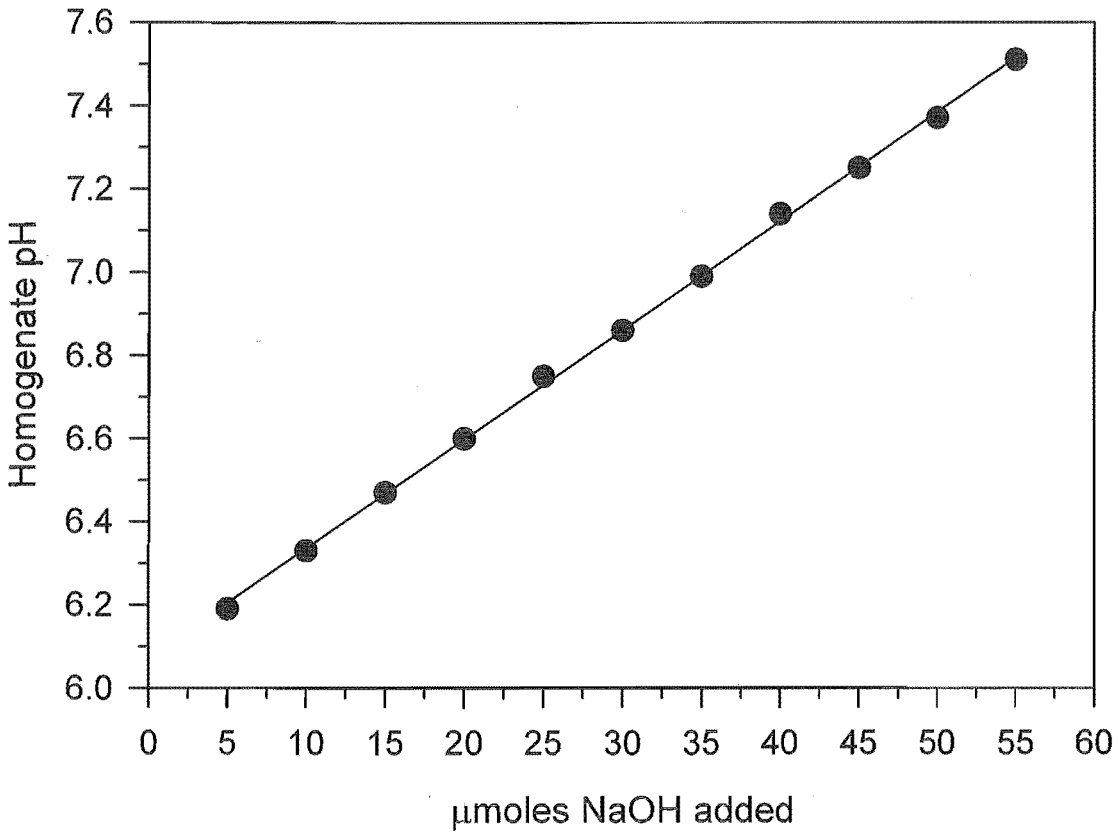

Figure 3.15: *A typical titration of a rested mullet white muscle homogenate with NaOH. The titration is described by the linear regression equation:* $y = 0.026x + 6.08$, $r^2 = 0.99$. *In this case the buffering capacity was calculated to be 76.92 Slykes.*

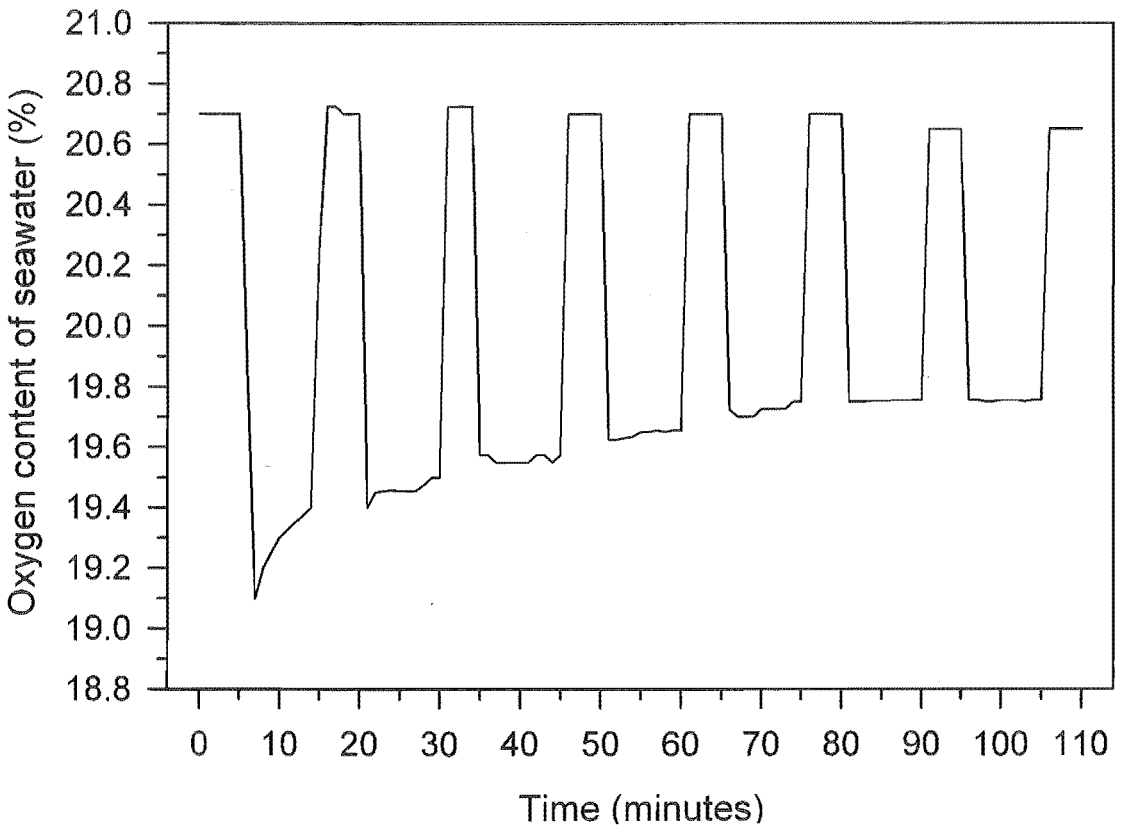

Figure 3.16: *A typical chart-recorder trace during the measurement of the rested oxygen consumption rate in an anaesthetised mullet. After 5 min of measuring the in-flowing water the out-flowing water was diverted through the dissolved oxygen probe and measured for 10 min. Measurements for oxygen consumption were taken toward the end of the trace when the out-flowing water oxygen content was stable.*

3.5 DISCUSSION

3.5.1 Husbandry and handling

The objective of the study was to compare and characterise the PM metabolism in rested and exercised mullet. It was important to establish a homogeneous population of tank rested animals that could be manipulated during experimentation and then compared with a true control. As peri-mortem fatigue alters the physiological and biochemical state of the WM, every attempt was made to eliminate handling and sampling artefacts in the rested fish. Being able to rear the fish under controlled conditions with adequate aeration, water supply and diet, allowed the author to observe the fish and be confident that they were indeed rested. Capture lesions healed quickly, the fish ate well and grew at a steady rate to the normal adult size for yellow-eye mullet.

Good animal husbandry practices, along with the use of minimal concentrations of an aquatic anaesthetic (AQUI-S^{TM} Plus) resulted in fish that were in a physiological state as close to resting as possible when harvested.

The anaesthetic concentration used to anaesthetise the mullet was higher than the recommended dose for salmonids (17 mg/L). The dose for mullet was determined in preliminary investigations and it was found that concentrations above 30 mg/L (dosage used in the current study) resulted in depressed post-harvest WM pH measurements. In all fish the brain and spinal cord was ablated using the traditional Japanese method of iki-jime. This ensured that the fish were killed quickly and that the nervous system was isolated from other organs. This method has often been characterised as destroying the medulla oblongata, the part of the hindbrain responsible for most of the autonomic reactions (Harada 1988). However, the exact anatomy has yet to be characterised in yellow-eye mullet.

3.5.2 Methodology

Exercise treatment

The exercise treatment was by no means designed to result in an exhausted state. Exhaustion is a highly defined state described by Dobson & Hochachka (1987). In the current study the exercise treatment was used to demonstrate the benefits of minimising

peri-mortem fatigue on the PM metabolism. The focus of this part of the study was not on "stress" per se, in as such that the "stress reaction" (activation of the hypothalamic-pituitary interrenal axis, Donaldson 1981) was not monitored in the fish after the forced exercise event. If the fish had been allowed to fully recover from the exercise bout other reactions such as increased haematocrit, and catecholamine and cortisol release (so called "stress reactions", Wardle 1981) would certainly affect the physiology of the fish in a way as to promote recovery. In this study, it was assumed that the principal effect of the exercise treatment on the PM WM was muscle fatigue and that the stress responses, primarily designed to aid muscle recovery, would have had little impact on the energetic status of the muscle. The true effects of these stress responses on the PM WM, free from the major confounding effect of peri-mortem exercise, have yet to be determined.

Mullet that had been forced to exercise prior to exposure to the anaesthetic became anaesthetised much faster than rested animals. As AQUI-S^{TM} Plus is an inhalation anaesthetic it is taken up into the fishes circulation via the gills, and possibly through the skin. The increased respiratory/ventilation rate associated with the forced exercise resulted in the mullet taking up the anaesthetic more rapidly than rested fish. This phenomenon has also been observed in exercised chinook salmon (O. tshawytscha) and snapper (P. auratus; Jerrett et al. unpublished data).

Cut-surface pH measurement

The initial, post-harvest WM cut-surface pH for rested mullet was considerably greater than intracellular values reported in the literature. Normoxic intracellular muscle pH (determined by ^{31}P-NMR) for carp and goldfish acclimated to 20 °C was found to be 7.36 ± 0.04 and 7.36 ± 0.05, respectively (van den Thillart et al. 1989), and 7.35 in tilapia acclimated to 20 °C (van Waarde et al. 1990), compared with 7.68 ± 0.03 for winter rested mullet in the current study. It was believed that the cut-surface pH mainly measures extracellular pH, with some contamination from intracellular fluid. Measurements of extracellular pH (arterial plasma pH) in rainbow trout have been reported to be 7.95 (Wang et al. 1994a) and in Atlantic cod (Gadus morhua) pH 7.6 (Berenbrink and Bridges 1994). In those same studies the intracellular pH (pH_i) of the red blood cells was reported as 7.23 in rainbow trout and ~7.22 in Atlantic cod. It was possible that the transverse cut across the fillet would release mainly extracellular fluid

surrounding the muscle fibres as well as some intracellular fluid from the fibres. It was thought that this was the reason why the cut-surface pH measured in the current study was somewhat higher than those reported for intracellular pH in the WM. It was not certain what, precisely, the cut-surface pH measures but it was very reproducible.

Jerrett et al. (1996) introduced the technique of WM cut-surface pH measurement in combination with anaesthetisation of rested (20-24 h of rest after dip-netting) salmon (O. tshawytscha) with AQUI-S^{TM}, reporting a resting muscle pH of 7.38 ± 0.05 (15.3–17.0 °C). The same techniques were also used in this study but with a much longer resting period prior to anaesthetisation (fish were only disturbed for feeding and cleaning of the tank, and were never dip-netted). Thus a higher resting WM pH was probably achieved due to conservative handling practices, but it was also possible that there were species differences.

3.5.3 Post-mortem rested and exercised, anaesthetised mullet

Blood measurements

The blood pH in rested mullet (acclimated to winter temperatures) was similar to those reported in flounder held at 9 °C (7.80, Milligan & Wood 1987) and rainbow trout held at 10 °C (7.85 Wood et al. 1990). In comparison with rested mullet, the large drop in blood pH and rise in blood lactate in the exercised fish (Fig. 3.2) reflected a metabolic acidosis, and likely respiratory acidosis, that had occurred during peri-mortem fatigue (for an excellent review of acid-base disturbances see Wood 1991). Although the exercise treatment was not designed to exhaust fish (only to show a disturbance) the blood pH in these fish was much lower (7.27 ± 0.05) than those reported in studies where fish were exercised to exhaustion by swimming (Snapper pH 7.44 at 19 °C, Jerrett et al. unpublished data; Rainbow trout pH 7.53 at 18 °C and pH 7.7 at 5 °C, Kieffer et al. 1994; Rainbow trout pH 7.52 at 14 °C, Pagnotta et al. 1994; Rainbow trout pH 7.5 at 15 °C, Wang et al. 1994a). Studies investigating how rainbow trout recover from exercise have shown that immediately after exhaustive swimming the arterial plasma Po_2 is lowered and the arterial plasma Pco_2 is increased (Milligan & Wood 1987; Wang et al. 1994a; Wood et al. 1990). In the current study holding the fish out of water to exercise them would probably have resulted in the Pao_2 dropping even lower than after exhaustive swimming due to the difficulty in extracting oxygen from air.

Inability to release CO_2 to the water would have also increased the $Paco_2$ beyond normal exhaustive levels. Following the forced exercise the fish were placed in seawater for anaesthetisation potentially allowing the fish to extrude some of the acid load through the gills. However, due to the rapid anaesthetisation of the exercised fish (~10 min) and the depression of ventilation that accompanies anaesthesia, any recovery from the respiratory and metabolic acidosis would have been minimal. Along with the protons released from lactate production and ATP hydrolysis in the WM (Wang et al. 1994b), the blood pH was driven lower than would normally occur in fish that are swum to exhaustion.

The increase in blood lactate concentration (Table 3.2) due to forced exercise was typical of the response observed in salmonids (Milligan & Wood 1986) which suggested that mullet also released some lactate generated in the WM into the blood stream rather than retaining all of it in the muscle for in situ metabolism. There were two very high concentrations of blood lactate measured in the exercised fish (Fig. 3.2), however, the blood pH did not drop to levels predicted by the regression equation. It has been shown that active, pelagic fish (e.g. trout, dogfish, tuna) may release large quantities of lactate into the circulation such that the lactate load considerably exceeds the metabolic acid load in the blood (Wood 1991). It is thought that the movements of lactate and H^+ out of the WM into the blood are dissociated, i.e. high levels of lactate in the blood may not directly coincide with low blood pH. A possible explanation for the two very high blood lactate concentrations in the exercised mullet.

The increased levels of glucose in the blood after exercise (Table 3.2) was indicative of catecholamine release into the circulation (Mazeud & Mazeud 1981; Wood 1991). Stress-related hyperglycaemia has been reported in many species of teleost and is mediated mainly by the effects of catecholamines on glucose release from the liver (main carbohydrate store) with adrenaline being more potent than noradrenaline (Wendelaar Bonga 1997).

Post-mortem white muscle pH profile

When mullet were exercised prior to harvesting the initial WM pH was ~0.5–0.6 pH units lower than winter acclimated rested fish (Fig. 3.4). In studies where rested O. tshawytscha WM were electrically exhausted prior to storage the WM pH values were

considerably lower than pH 7.0 and were typically in the range of pH 6.3 to 6.6 (Jerrett et al. 1996; Jerrett & Holland 1998). This range was similar to the ultimate pH observed in the mullet WM during PM storage (Fig 3.4). It demonstrated that the exercised WM in the current study was by no means close to the "exhausted" state described by Dobson & Hochachka (1987), or produced by the PM electrical exhaustion treatment of Jerrett et al. (1996) and Jerrett & Holland (1998). The variation in initial WM pH values was high in the exercised fish with values ranging from pH 6.88 to 7.39 compared with 7.58 to 7.74 in winter rested fish. This reflected the high and variable blood lactate levels in exercised fish (6.8 to 17.2 mmol/L; Fig. 3.2) and highlighted the increased "metabolic consistency" of the rested fish. Forced exercise causes a profound disturbance of ionic, osmotic and fluid volume homeostasis (Wood 1991).

Standard oxygen consumption in rested and exercised, anaesthetised mullet

In the two mullet (sampled in winter for metabolic rate measurement) that had been forced to exercise prior to anaesthesia the average $\dot{M}O_2$ was 75.0 ± 4.0 μmol/min/kg body mass, compared with 26.1 ± 2.5 μmol/min/kg body mass in the 5 rested fish sampled, i.e. the exercised $\dot{M}O_2$ was ~2.8 times that in rested fish. In the exercised fish, the oxygen consumption rate in the WM was estimated to be 45 μmol/min/kg muscle mass (0.045 μmol/min/g muscle mass). In ATP equivalents (6 ATP for every O_2) the exercised mullet used ATP at a rate of 270 μmol ATP/min/kg muscle mass (0.27 μmol ATP/min/g muscle mass). The average weight of the 2 exercised fish was 84.7 ± 12.1 g. As ~60% of the body mass was WM (see Results Section 3.4.5: Resting and post-exercise oxygen consumption) this would equate to ~50 g of WM. Therefore, if the exercised WM consumed ATP at a rate of 0.27 μmol ATP/min/g muscle mass that equated to ~13.5 μmol ATP/min in all the WM. As there was ~7.5 μmol/g of ATP stored in the WM (see Results Section 3.4.3) equating to ~375 μmol in all the WM the mullet could consume ATP at this rate for ~28 min before needing to generate ATP by anaerobic means. However, ATP can be depleted in the WM in a matter of minutes during burst exercise. Moyes & West (1995) reported an ATP utilisation rate in the WM of shorthorn sculpin (Myoxocephalus scorpius) at maximal work levels of 90-126 μmol/min/g muscle mass, three orders of magnitude higher than that calculated from the

$\dot{M}O_2$ in the current study (0.27 μmol ATP/min/g muscle mass). It would appear that calculating ATP consumption rates from $\dot{M}O_2$ grossly underestimated the true rate.

Overall, the large increase in metabolic rate due to the forced exercise prior to anaesthetisation and the consequent disturbance of ionic, osmotic and fluid volume homeostasis would have increased the rate of ATP degradation in the WM along with the rates of all other chemical changes (Bate-Smith & Bendall 1956). Even though the high energy phosphate stores could have been mostly depleted due to exercise, the WM pH still decreased to low levels during PM storage. This observation suggested that there was still some potential to generate ATP in the WM after harvesting, i.e. glycogen was still present.

Ultimate pH of white muscle from rested and exercised anaesthetised mullet

The WM pH of the exercised mullet decreased to a slightly lower ultimate pH than rested fish. This result has also been reported elsewhere (Fletcher et al. 1997; Love 1980; Thomas et al. 1999). Love (1980) suggested that the variations in ultimate pH may be due to some glycogen being hydrolysed to glucose rather than converted to lactic acid. However, this theory has been disputed by Thomas et al. (1999) and the reason for lower ultimate pHs in exhausted WM remains unclear. Another possibility relates to the amount of glycogen stored in the WM. In the current study exercised mullet had higher HSIs than rested fish suggesting that more glycogen may have been in the WM of exercised fish. Therefore the WM pH could have been able to decrease to lower levels due to more lactate being produced. It was unfortunate that the WM substrates and metabolites were not measured in these fish prior to the harvest so this theory could either be confirmed or discounted. The ultimate pH in the winter rested mullet sampled in 1998 (Fig. 3.4) was very similar to that in the exercised mullet. This suggested that the winter 2001 rested mullet did, indeed, have lower stores of glycogen in the WM resulting in a higher ultimate pH (less lactate produced).

Overall, it was clear that harvesting fish in their rested state minimised respiratory and metabolic disturbances and as a result of this reduced the rate of PM changes in the WM.

3.5.4 Winter acclimated rested mullet: different sized fish

Blood measurements and physical condition

Rested mullet sampled in winter 1998 had slightly lower blood pHs than those sampled in winter 2001 (Table 3.3). As described above (see section 3.4.2), this was most likely due to peri-mortem exercise as indicated by the higher blood lactate level and slightly higher blood glucose concentration. The relationship between mixed venous blood pH and blood lactate concentration was the same in the different sized fish as it was in the rested and exercised fish (see legends of Figs 3.2 & 3.6). Even though the winter 2001 mullet were ~80 g heavier than the 1998 mullet, the CF and HSI for the 2001 fish were lower. The age of the 2001 fish was ~3.5 years and the age of the 1998 fish was ~2.5 years. Yellow-eye mullet mature after 3 years and can live for 5 to 7 years (Paul 2000). It was possible that the older fish were losing condition due to their age.

Post-mortem white muscle pH profile

Even though there was a considerable size and age difference between the two groups of fish there was no difference in WM pH immediately after harvest and also during PM storage (Fig. 3.7). Size and age, therefore, did not appear to alter PM metabolism significantly in yellow-eye mullet. This was somewhat surprising as metabolic rate usually decreases with an increase in body size (Schmidt-Nielsen 1993). It may be that there was not a large enough difference in body size to detect PM differences (only ~80 g weight difference). However, in the WM of larger fish, anaerobic enzymes have been reported to show increased activity in larger fish (Goolish 1995). It has been reported that large rainbow trout have higher muscle lactate concentrations and also produce lactate at a faster rate when chased to maximal activity (Somero & Childress 1980). They also display positive allometry for WM buffering capacity. Thus, even though the two groups of fish used in the current study were different sizes, the positive scaling effect on anaerobic processes may have resulted in no difference to the WM pH measured during PM storage.

3.5.5 Post-mortem metabolic characterisation of rested white muscle

Blood pH and pre-storage white muscle pH: effect of acclimation temperature

The blood pH for the rested mullet sampled in December 1998 (summer 20.8 ± 0.1 °C) was similar to that measured in the rested mullet sampled in September 1998 (winter 11.5 ± 0.1 °C; see Table 3.3). However, the September 1998 fish had higher blood lactate levels suggesting that the fish may have exercised prior to harvesting and will be discussed in the next section. Studies investigating the effects of temperature on acid-base regulation in fish report that the change in blood pH with temperature has a linear relationship with a slope of typically –0.015 pH units/°C (-0.0132 pH units/°C in Channel catfish; Cameron and Kormanik 1982; Heisler 1984). As there was a 9.3 °C difference in temperature between the summer 1998 and winter 1998 acclimated mullet it was expected that the blood pH in summer rested mullet would be 0.12 pH units lower than the winter mullet. This was not the case in this study and the discrepancy may be explained by the higher blood lactate concentration in the winter mullet, resulting in a depressed blood pH. The blood pH of winter rested mullet sampled in July 2001 (7.82 ± 0.06; ambient temperature 9.9 ± 0.2 °C) was similar to those reported in flounder held at 9 °C (7.80, Milligan & Wood 1987) and rainbow trout held at 10 °C (7.85 Wood et al. 1990). These fish also had lower blood lactate levels than the 1998 winter mullet with concentrations more similar to the summer 1998 mullet (see Tables 3.2 & 3.3). The difference in ambient temperature between summer 1998 and winter 2001 was 10.9 °C. The difference in blood pH between 1998 summer and 2001 winter mullet was 0.13 pH units, not very different from the expected value calculated from the pH/temperature relationship (0.14 pH units).

The effect of acclimation temperature was also observed in the rested WM pH when the rested summer 1998 acclimated fish and winter 1998 acclimated fish (Fig. 3.7) were compared. There was a significant difference ($P < 0.05$) in the pre-storage WM pH values (7.61 ± 0.02 in summer WM and 7.74 ± 0.03 in winter WM). These values were consistent with the predicted increase in pH with a decrease in temperature, seen in the blood pH.

Hypoxia during anaesthetisation may depress blood pH

Some of the individual summer rested mullet blood pHs were lower than expected for rested fish. This was possibly due to the fish becoming hypoxic during the anaesthetisation process (rate of operculation slows and therefore washout of CO_2 slower), rather than due to fatigue since the blood lactate concentration remained low. This further highlighted the importance of knowing the characteristics of rested fish and being able to recognise when "rested" fish may have been slightly fatigued prior to harvest (i.e. the winter 1998 rested mullet had slightly higher blood lactate levels).

Pre-storage metabolite levels in rested white muscle

Resting metabolite levels in the WM were similar to those reported in the literature for salmonids (rainbow trout review by Milligan 1996; Dobson et al. 1987). Resting levels of lactate were reported to be between 0 and 11 μmol/g muscle mass which was in agreement with the levels found in the rested mullet WM in the current study (12.7 ± 1.0 μmol/g muscle mass; Fig. 3.9a).

Pre-storage ATP levels in rested mullet WM (7.4 ± 0.7 μmol/g muscle mass; Fig. 3.9b) were slightly higher than those reported for rainbow trout (5.74 μmol/g muscle mass 13 °C, Thomas et al. 1999; 4.99 ± 0.26 μmol/g, Dobson et al. 1987) and Atlantic salmon (4.51 μmol/g muscle mass 13 °C, Thomas et al. 1999). However, Lowe (1992) recorded an ATP concentration of 7.16 ± 0.89 μmol/g muscle mass in summer captured snapper (Pagrus auratus), a concentration similar to that found in this study. Rested harvesting and conservative handling are the most likely reason for the conservation of the ATP store. However, species differences along with acclimation temperature may have also influence the levels.

Resting P_i values were slightly higher in rested mullet compared with rainbow trout (22.9 ± 2.1 μmol/g muscle mass; Fig. 3.9c, compared with 15.22 ± 0.56 μmol/g muscle mass, 4-6 °C, Dobson et al. 1987). This may have been a species difference or some of the high energy phosphates (ATP and PCr) may have been degraded during handling in the case of summer acclimated mullet. The later hypothesis was unlikely as there were high levels of ATP in the rested WM.

Although there were problems with the free creatine assay (readings above 20 μmol/g muscle mass were not reliable), the pre-storage concentration in the WM was below 20 μmol/g muscle mass (12.6 ± 1.0 μmol/g muscle mass). Much higher levels of free creatine (31.50 ± 0.87 μmol/g muscle mass) were reported by Dobson et al. (1987) in resting rainbow trout (4-6 °C). However, the total amount of PCr present in rainbow trout was thought to be 40 μmol/g muscle mass (from ^{31}P-NMR analysis), which is somewhat higher than reported in other species (20–30 μmol/g muscle mass, van den Thillart & van Raaij 1995). The high level of free creatine in resting rainbow trout suggested that either the fish were not truly rested or that the WM sampling procedure resulted in the breakdown of PCr to creatine. Assuming that ~30 μmol/g muscle mass of PCr was present in the WM of mullet in this study, it was possible that approximately 25 to 50% of the PCr was broken down to creatine during the sampling process in the current study.

The pre-storage glycogen concentration in rested WM (37.6 ± 5.6 μmol/g muscle mass) was in agreement with those reported by Moyes & West 1995 (40 μmol/g muscle mass) for resting salmonids.

Post-mortem pH profiles in rested white muscle

The WM pH in rested mullet decreased steadily over the first ~27 h of PM storage, after which the ultimate pH was reached (Fig. 3.8). In other similar studies carried out in the author's laboratory (in particular the investigation into the effects acclimation temperature and storage temperature had on the PM metabolism of mullet WM Law & Jerrett 1996 unpublished results; see Methods: 3.6 Choice of Storage Temperature), the decrease in pH of WM at the same storage temperature and storage time (10.0 °C, ~21 h) was similar compared with the current study. Interpolating the data on Fig. 3.1, mullet acclimated to summer temperatures stored at 10.0 °C would reach a pH of ~6.7 after 21.6 h compared with a pH of ~6.65 in rested WM in the current study (Fig 3.8).

Possible effect of acclimation temperature on post-mortem pH profile in rested white muscle

As in the case of blood pH measurement in summer and winter acclimated fish, there may have also been some differences in the PM WM pH profiles between fish acclimated to different temperatures. The decrease in pH over the first ~12 h of PM

storage was faster in winter acclimated fish compared with summer acclimated fish. However, the ultimate pH of the WM was reached at the same time in both summer and winter acclimated mullet. As the PM metabolites were not measured in the winter acclimated fish it was unclear what effect acclimation temperature had on PM metabolism.

Post-mortem metabolism in rested white muscle

In the Law & Jerrett (1996 unpublished results) study, even though the pH was similar after the same PM storage time in the current study, there was a substantial difference in WM [lactate] (Fig. 3.9a). In the summer acclimated mullet of the Law & Jerrett study the WM [lactate] was in the range of 25-30 μmol/g muscle mass after 21.6 h (WM pH ~6.7) if it were stored at 10 °C (Fig. 3.1), whereas in the current study it was ~60 μmol/g muscle mass (WM pH ~6.65) at a similar time PM. Even taking into account the difference of 0.05 pH units in the WM this would only equate to ~3 μmol/g of lactate, not the 30 μmol/g difference in lactate concentration between the two. The CF and HSI of the summer acclimated mullet sampled for the Law & Jerrett study were significantly lower than those sampled in the current study (CF of 0.99 ± 0.02 compared with 1.40 ± 0.04; $P < 0.05$; HSI of 1.53 ± 0.08 compared with 2.93 ± 0.41; $P < 0.05$). This suggested that the glycogen stores in the WM of the Law & Jerrett mullet were not as high as those in the mullet of the current study. It was possible that the lower glycogen levels could have resulted in a lower WM lactate concentration, however, in the Law & Jerrett study when mullet were stored at 2 and 18 °C lactate levels rose to over 50 μmol/g muscle mass after 21.6 h (Fig. 3.1). This point will be discussed further later in this section with regards to glycogen.

When the mullet were harvested for the Law & Jerrett study they were stored in the round in RSW (refrigerated seawater). This would have cooled the WM considerably faster than the fillets held in oxygen in the current study. The rapid cooling may have slowed the metabolism of the WM resulting in the decreased demand for ATP production, in turn slowing the rate of lactate production. However, this should have resulted in higher WM pH levels than were measured. The reason for the discrepancy in WM lactate levels between the two groups of mullet remains unclear, but the physical condition of the fish may have played a significant role. It is possible that the buffering capacity of the mullet in the Law & Jerrett study was lower (due to their

poorer condition) resulting in an earlier inhibition of glycolysis by acidotic conditions during PM storage.

During PM storage [ATP] in the rested WM decreased to a minimum in a similar pattern to WM pH, and inversely to the increase in WM lactate (Fig. 3.9b). This indicated that ATP levels in the WM could not be defended by the hydrolysis of PCr ~12 h post-harvest. Due to the ischemic condition of the WM, any energy required by the cells would have needed to be produced via anaerobic processes, hence the increase in lactate.

Free creatine in the WM increased during PM storage in the rested WM indicating the hydrolysis of PCr was used for the generation of ATP (Fig. 3.9d).

Although the WM glycogen levels were quite variable in the rested WM, the amount of glycogen did not drop to the lowest levels until after ~35 h storage (Fig. 3.9e). This suggested that glycogen may not have been the only substrate available to the WM to generate ATP. After ~35 h storage there was still substantial amounts of glycogen (~15-20 μmol/g muscle mass) in the WM even though the WM had reached its ultimate pH, maximum lactate levels and was depleted of ATP. It was uncertain whether there was a threshold concentration of glycogen in the WM that once reached the remaining glycogen was not available for further supply of ATP. Alternatively, the biochemical conditions in the cell may prevent the continuation of anaerobic glycolysis by inhibiting key enzymes, e.g. phosphofructokinase being inhibited by low pH.

In the temperature storage study carried out by Law & Jerrett (1996), the fish were of a similar length compared with rested mullet in the current study (258 ± 5 mm; n = 26; compared with 247 ± 7 mm; n = 5, respectively), however, their mean weight was less (165.2 ± 7.9 g; n = 26; compared with 214.8 ± 23.3 mm; n = 5). A study by Ferguson et al. (1993) found that glycogen levels in the WM scaled positively with body size and therefore increase in lactate concentration was greater in larger fish. Somero & Childress (1980) also found that glycolytic enzymes (lactate dehydrogenase and pyruvate kinase) also have higher activity in larger animals (See Section 3.5.4: Different sized fish). This was also supported by the finding that lactate concentration in the rested WM prior to storage was lower in the smaller fish (5.8 ± 0.4 μmol/g muscle mass, n = 6; compared with 12.7 ± 1.0 μmol/g muscle mass, n = 5). The fish in

the current study also had a much higher HSI compared to those in the temperature storage study (2.93 ± 0.41; n = 5; compared with 1.53 ± 0.08; n = 25), indicating that glycogen stores may have been higher, therefore allowing more lactate to be generated. As discussed in Section 3.5.4 (Different sized fish) the buffering capacity of the WM would have also increased with body size and therefore the pH profile during PM storage was similar. This may partially explain the discrepancy in WM lactate levels between the two groups of mullet discussed.

Unfortunately the metabolites measured in the rested WM during PM storage were not measured in the exercised WM. However, there is extensive literature on the subject indicating that after forced exercise, high energy phosphate stores (ATP and PCr) were likely to be depleted, along with increased lactate and P_i concentrations (Dobson & Hochachka 1987; Thomas et al. 1999; Wang et al. 1994a). In the present study, the relationship between pH and lactate (Fig. 3.10) indicated that at a pH of 7.14 ± 0.06 (initial WM pH of exercised fish) the WM [lactate] would have been ~30 μmol/g muscle mass, indicative of anaerobic production of ATP. Thus, the 'ATP potential' in exercised fish would have been much lower prior to going into storage than rested fish.

3.5.6 White muscle pH measurement is a good predictor of the physiological state of the muscle

The WM pH measurement was a good predictor of the physiological state of the WM and had strong relationships with lactate and ATP over the pH range measured (Figs. 3.10a & 3.11a). There was also a strong relationship between WM lactate and ATP concentration (Fig. 3.12). This was not surprising as ATP depletion is inherently linked to lactate accumulation through the need to continually generate ATP. WM pH measurement was not such a good predictor of WM [P_i] (Fig. 3.13a) because P_i could still be generated through breakdown of ATP and ADP even when the ultimate pH had been reached. Creatine and glycogen levels were not well predicted by WM pH, however, the increase in creatine and decrease in glycogen WM concentration was consistent with the decrease in [ATP] and the use of anaerobic metabolism for ATP re-synthesis. Overall, measurement of WM pH alone gave a very rapid, simple and accurate description of the physiological state and time-course of glycolysis in the WM during PM storage.

3.5.7 Significance of buffering capacity

The capacity of the intracellular fluids to buffer acidic end-products, such as lactic acid, is essential in the muscle cell. Castellini & Somero (1981) stated "without adequate buffering capacity, even the high activity of glycolytic enzymes and large stores of glycogen may not be sufficient to allow a muscle to conduct either intense, short-term bursts of anaerobic glycolysis or more prolonged, low-level periods of metabolism under low oxygen conditions". The latter of those two scenarios was more appropriate to the current study as it was desired that the ischemic WM preparation could generate energy and stay functioning for as long as possible. The buffering capacity in the WM of rested mullet acclimated to summer temperatures was 78.7 ± 3.6 Slykes. This result was slightly higher but in agreement with values reported by Castellini & Somero (1981) for actively foraging pelagic teleosts. Less active species have correspondingly lower buffering capacities. With a relatively high buffering capacity it would be expected that the lactate dehydrogenase activity of the WM would also be high to match the activity level of the mullet. Pörtner (1990) suggested that the method used to measure non-bicarbonate buffering capacity in the Castellini & Somero study (muscle homogenate titrated with strong base) over-estimated the buffering capacity. This was due to metabolic processes occurring during titration, particularly accumulation of P_i leading to an increase in buffer value. This would be of concern in the current study due to the high ATP content of the rested WM. It could be assumed then that the buffering capacity of the WM at rest would be lower than after hydrolysis of PCr and ATP due to the low levels of P_i.

Even though the non-bicarbonate buffering capacity of the WM may have been over-estimated it appears very well matched to the amount of lactate produced. The buffering capacity of rested mullet WM was 78.7 ± 3.6 Slykes and the amount of lactate accumulated during PM storage was ~70 μmol/g muscle mass (Fig. 3.9a). This amount of lactate production resulted in a change in WM pH from ~7.6 to ~6.4 (a change of ~1.2 pH units, Fig. 3.8), equivalent to a $[H^+]$ of 3.58 x 10^{-4} mmol/L (pH = -logK_a). Assuming that a WM lactate concentration of 70 μmol/g muscle mass is equivalent to ~70 mmol/L, the amount of hydrogen ions that would dissociate from this concentration would be 3.37 mmol/L (for calculation see Appendix 2). This would equate to a pH of 2.47. The concentration of H^+ from the dissociated lactate was four orders of magnitude

greater than the calculated H^+ change from the WM pH measurements during PM storage. This clearly showed that the buffering capacity of the WM was high over the pH range faced by the WM during PM storage and could "absorb" large amounts of H^+. Fig. 3.10b shows the relationship between $[H^+]$ (calculated from the WM pH) and WM lactate content. Concentrations of lactate up to ~50 μmol/g muscle mass resulted in very little change in $[H^+]$ of the WM. These H^+ concentrations corresponded to WM pHs above ~6.9. During burst exercise the WM pH rarely falls below ~6.8 (O. tshawytscha and P. auratus Jerrett et al. unpublished data). It was then not surprising that the majority of the buffering capacity of the WM was effective above pH 6.8 (below ~1.6×10^{-7} mol/L $[H^+]$, Fig. 3.10b).

When a fish is swimming aerobically the rate of flux through glycolysis would be relatively low due to the efficiency of aerobic ATP generation. During burst exercise the WM becomes hypoxic and ATP stores are depleted. The increased levels of ADP activates PFK (a key regulator of glycolysis) to increase flux through glycolysis to increase energy production. The increased levels of fructose 1,6 bisphosphate (See Chapter 1, Fig. 1.2) would result in feed-forward activation of pyruvate kinase which increases pyruvate production. High levels of pyruvate drive the production of lactate through lactate dehydrogenase (Mathews & van Holde, 1990) which has to occur rapidly as the efficiency of ATP generation through this reaction is poor. The production of lactate also produces H^+ and reduces the pH of the WM. Although lactate levels in the WM reached ~70 μmol/g muscle mass during PM storage, after burst exercise in O. tshawytscha and P. auratus lactate levels in the WM were only 25 μmol/g mass with a corresponding WM pH of ~6.95 (Jerrett et al. unpublished data). It would appear that maintaining the WM at a neutral pH during burst exercise is the optimum for anaerobic ATP generation through glycolysis. If the WM pH did drop below ~6.8 during burst exercise it is possible that the likelihood of recovery would be much less as the low pH would inhibit several enzymes necessary for glycolysis (such as PFK, Dobson et al. 1986). Inhibition of glycolysis would consequently prevent the fish returning to homeostasis.

3.5.8 Estimations of metabolic rate

Bate-Smith & Bendall (1956) described an inverse relationship between muscle pH and lactate (also described in the current study). From that correlation they could calculate the "time-course of glycolysis" (re-synthesis of ATP) using the equation:

$$\frac{\Delta \text{lactate}}{\Delta \text{pH}} \qquad \text{(sic)}$$

Their calculation (converted into ATP) was 97.5 μatom ATP-P/pH/g muscle mass (95 μmol ATP/pHunit/g muscle mass). Using the lactate/pH relationship in the current study (Fig. 3.10) the value was:

$$\frac{\Delta \text{lactate}}{\Delta \text{pH}} = 54.0 \text{ μmol lactate/pH unit/g muscle mass (x 1.5 lactate :3 ATP)}$$

$$= 81.0 \text{ μmol ATP/pH unit/g muscle mass}$$

The value calculated for the rested mullet WM was lower than that calculated for the rabbit psoas muscle most likely due to the increase in metabolic rate in warm-blooded mammals. It was not substantially different, however, due again to the use of the same metabolic pathway for ATP re-synthesis.

The oxygen consumption measured in the anaesthetised rested mullet (26.1 μmol O_2 /min/kg body mass) was similar to those reported for inactive, bottom-dwelling species such as turbot ($\dot{M}O_2$ = 22 μmol O_2 /min/kg body mass, Maxime et al. 2000) and starry flounder ($\dot{M}O_2$ ~17 μmol O_2 /min/kg body mass, Milligan & McDonald 1988). In more active species where the oxygen consumption is measured while the fish is swimming, the $\dot{M}O_2$ is slightly higher, e.g. coho salmon $\dot{M}O_2$ ~42 μmol O_2/min/kg body mass (Milligan & McDonald 1988). The $\dot{M}O_2$ of mullet that had been exercised prior to anaesthetisation was ~2.8 times that of rested mullet, indicative of a significant oxygen debt.

This magnitude of difference in oxygen consumption between rested and exercised, anaesthetised mullet was very similar to the difference in rate of H^+ accumulation in the WM of rested and exercised mullet during PM storage (~2.6 times,

see Fig. 3.3). Studies measuring the recovery from exhaustive exercise have found that there are two components to the excess post-exercise oxygen consumption (EPOC): a 'fast' component where 83% is attributed to ATP and PCr resynthesis, recharging O_2 stores and increased cardioventilatory work, and a 'slow' component (Wood 1991). It was originally thought that the slow component was the metabolism of lactate back to glycogen but studies have now shown that only 25% of the slow component is explained by lactate clearance (Scarabello et al. 1991). It is now thought that the costs of altered membrane transport processes associated with re-establishing ion, acid-base and fluid homeostasis maybe important contributors to EPOC. It has also been demonstrated in mammals that EPOC is not affected by short exercise duration (i.e. does not change after exercise bouts ranging from 5-60 s; Baker & Gleeson 1998). However, this does not appear to be the case in ectothermic vertebrates. In the desert iguana (Dipsosaurus dorsalis) EPOC duration increased with activity duration (5-60 s) and was sensitive to catecholamine release post-activity (Nedrow et al. 2001). In the current study it was probable that the recovery of the oxygen debt incurred by the exercised mullet (~70 min to reach an $\dot{M}O_2$ not significantly different to rested levels) was mainly due to ATP and PCr resynthesis (fast component of EPOC). It was uncertain how long it would take exercised mullet to re-establish ion, acid-base and fluid homeostasis. From literature reporting metabolite recovery from exercise (i.e. ATP, PCr, lactate) it could be suggested that this "slow" component of EPOC may take up to 12 h to return to resting levels (Milligan 1996; Milligan & McDonald 1988; Moyes et al. 1993; Turner et al. 1983).

Pre- and post-mortem respiration rates

As mentioned in the "Results" Section 3.4.5 (Resting and post-exercise oxygen consumption) the $\dot{M}O_2$ of rested anaesthetised fish was not converted into an ATP consumption rate as oxygen consumption in the WM at rest would be minimal compared with the rest of the body. Therefore, it was difficult to compare the pre-mortem respiration rate to those observed in the PM WM. However, assuming that when the fish was at rest the WM was consuming oxygen at a similar rate to the rest of the body the resting $\dot{M}O_2$ of the WM would be 60% of 26.1 ± 2.5 μmol O_2/min/kg body mass i.e. 15.7 ± 2.5 μmol O_2/min/kg muscle mass (0.016 ± 0.003 μmol O_2/min/g muscle

mass. Converting the oxygen to ATP equivalents (6 ATP for every O_2) the rate was 94.2 ± 15 μmol ATP/min/kg muscle mass (0.094 ± 0.02 μmol ATP/min/g muscle mass).

Moyes & West (1995) reported that if skeletal WM was recruited at maximal work levels then the expected ATP to be generated aerobically would be 60-84 μmol/min/g muscle mass. This is three orders of magnitude higher than the estimated AMR calculated in this study. However, at maximal work output the WM is actively contracting requiring a large amount of ATP to be supplied to it. In the current study, the ischemic WM was not required to actively contract during storage (apart from random firing of neurons), therefore the ATP demand would have mainly come from ion pumps (Na^+/K^+ ATPase) in the cell membrane attempting to maintain homeostasis (Hochachka 1997). When the whole animal is exercising, it uses oxidative phosphorylation to fuel a sub-maximal work rate. When ATP demand outweighs ATP supply and [ADP] and [P_i] increase, conditions favour glycolytic competition for both chemical species. Flux through the energy producing systems is already high and continuing to rise. In the rested isolated preparation that has intact ATP and glycogen stores the flux through anaerobic glycolysis reactions would be minimal due to the fish respiring aerobically prior to harvesting. Due to the low demand for ATP in the rested WM any "flare-up" in the anaerobic glycolytic rate would be minimised to a "dull-roar".

3.5.9 Questions to arise from these experiments

Although the experiments outlined in this chapter were carried out in order to better characterise the PM changes occurring in rested WM of the mullet several questions arose during these experiments. Primarily they were regarding how different factors may influence the PM metabolism of the WM.

When comparing the blood pH in the different groups of rested mullet it was noted that acclimation temperature had an affect on the value, in particular, in winter acclimated mullet the blood pH was higher. Also, in comparing the PM WM pH profiles, the pre-storage winter WM pH was higher in winter fish but the rate of decline in pH during storage was faster than in summer WM. The question then arises: if the temperature can affect the pH of the blood and the WM then how does this relate to PM metabolism?

When buffering capacity of the rested WM was measured it was carried out on winter acclimated fish. However, the PM measurement of WM metabolites and calculation of AMR was made on summer acclimated fish. The question arises as to whether the buffering capacity of the WM changes with acclimation temperature, and again, how does this relate to PM metabolism?

Similarly, the oxygen consumption rates measured in rested and exercised fish were carried out in the winter. In the winter fish are often more sluggish and therefore it could be expected that their O_2 consumption rate may also be slower. If all processes are slower at winter temperatures then could PM metabolism also occur more slowly?

These questions regarding acclimation temperature are further investigated in Chapter 7. The main focus of the thesis, however, was on significantly modifying the PM metabolic rate of the WM. The current chapter has shown that pre-mortem exercise greatly increased the rate of PM changes (as indicated by WM pH) by increasing the metabolic rate and by depleting the WM of precious energy stores even before PM storage began. Maintenance of these energy stores during PM storage, whether it be by slowing the rate of metabolism and therefore slowing the rate of utilisation of energy, or by allowing the WM to generate ATP via pathways that do not result it harmful end-products, appears to be the key in extending cell viability in the ischemic preparation. This will be the focus of the following chapter.

3.6 SUMMARY

Peri-mortem fatigue (exercise) resulted in large disturbances to blood acid/base balance along with acidification of the WM. This "metabolic disruption" lead to an increased rate of H^+ production during PM storage. Harvesting mullet in a rested state minimised any physiological disturbance to the blood and WM resulting in a substantial slowing of PM changes in the WM during storage. There was no difference in PM WM pH profile between winter acclimated mullet of different size. The buffering capacity of the WM appeared well matched for the amount of lactate that could be produced during PM storage. The estimated anaerobic metabolic rate of rested WM during PM storage was similar to the standard oxygen consumption ($\dot{M}O_2$) of rested anaesthetised mullet. Exercised mullet had an $\dot{M}O_2$ ~2.8 times that of rested fish. Overall, any level of

fatigue a fish experiences prior to harvesting resulted in highly varied levels of physiological disturbances to the acid/base balance of the blood and WM. The metabolic rate of the WM was increased with a concomitant reduction in the ATP potential. Mullet harvested in a rested state had a high WM ATP potential with little variation between fish. As a result the WM remained functional for much longer during PM storage.

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CHAPTER 4

The effect of supplementary hyperbaric oxygen supply on the post-mortem metabolism of rested, ischemic white muscle from yellow-eye mullet (Aldrichetta forsteri).

4.1 ABSTRACT

It has been shown that maintenance of ATP and glycolytic stores in the WM through rested harvesting was a critical factor in delaying the loss of PM functionality in the ischemic WM of yellow-eye mullet (Chapter 3). An investigation was made into extending the maintenance of ATP and glycolytic substrate levels in the ischemic WM preparation of yellow-eye mullet by allowing the tissue to respire aerobically. Hyperbaric oxygen therapy in mammals results in greater amounts of oxygen dissolved in the blood stream which promotes healing in compromised tissues. Preliminary experiments showed that anaesthetised mullet exposed to low hydrostatic pressure for a short period had higher WM pHs during PM storage with sensitivity to electrical stimulation being retained for longer compared with mullet not exposed to the treatment. As there were problems with this method, delivery of oxygen to an ischemic WM preparation was attempted. Various PM storage pressures and gas mixes were trialed with the greatest effect being shown by storage of ischemic WM at 620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%. This was then compared with tissue stored under normobaric conditions with humidified oxygen flow set to 50 mL/min ± 1.25%. A flow of oxygen through the storage chamber was found to be essential in providing the benefits of PM hyperbaric storage. Storage of the WM under hyperbaric conditions appeared to counter or mask the detrimental affects of PM storage at low temperatures (acceleration of acidification). PM changes in WM cut-surface pH were measured during storage along with key metabolites (lactate, ATP, creatine, P_i and glycogen). Storage of the WM under pressure at the appropriate temperature reduced the rate of pH decline by two-thirds of the rate exhibited by the normobaric control. The onset of ATP and glycogen depletion with its concomitant increases in tissue lactate, P_i and creatine was retarded/delayed by ~25 h. Possible reasons why the

hyperbaric WM changes from aerobic to anaerobic generation of ATP (even though substrate was not limiting) are discussed. We presumed that supplying the ischemic WM with oxygen enabled the tissue to respire aerobically for an extended period, maintaining ATP and glycogen levels in tissue. However, even with an oxygen supply the tissue still showed a slow reduction in pH resulting from anaerobic glycolytic generation of ATP.

4.2 INTRODUCTION

In well nourished fish, minimising peri-mortem fatigue using rested harvesting techniques will maintain endogenous levels of high energy phosphagens (ATP, PCr, etc) and stores of glycolytic substrates in the ischemic WM. The tissue can then enter PM storage with a high ATP potential (Chapter 3). Storage of the rested WM at a temperature that is optimal for reducing the rate of anaerobic metabolism has, so far, provided the maximum extension to the "life" of the PM tissue (Chapter 3, Jerrett et al. 2000).

During PM storage of the ischemic WM, energy for maintaining cellular homeostasis can only be supplied from endogenous ATP stores, PCr catabolism, or, once the high energy phosphagen stores have been largely depleted, through anaerobic glycolysis. Production of ATP via anaerobic glycolysis is a fast but inefficient process and results in the production of lactate. In WM the metabolic lactacidosis (drop in pH) produced by anaerobic glycolysis progressively and inevitably deepens and does little to sustain homeostasis. In contrast, aerobic production of ATP is highly efficient compared with anaerobic glycolysis and has less damaging end products.

When the WM is at rest (i.e. swimming at speeds where red muscle is dominant) it is most probable that its energy demand is supported aerobically (Moyes et al. 1989). However, the WM of teleosts is mainly used in burst, high intensity exercise. The muscle is poorly perfused and the high ATP demand during burst exercise cannot be met by mitochondrial oxidative phosphorylation. This is probably because of limitations in both oxygen delivery and mitochondrial density (Moyes et al. 1989). During recovery from exercise some of the lactate produced is often retained in the WM and is then re-synthesised to glycogen in situ (Milligan & Girard 1993). This process requires ATP and is produced via oxidative phosphorylation. Therefore, even though the WM is normally employed for burst exercise and supplies energy through anaerobic metabolism, it also has the capacity to respire aerobically to recover from the effects of exercise.

Rested WM with a high ATP potential could then be expected to be able to respire aerobically during PM storage if there was a continued supply of oxygen. As the ischemic preparation has no circulation, delivery of oxygen to the tissue would

depend upon diffusion. In humans in compromised states, such as trauma, oedema, diabetes and radiation sickness, healing is limited in hypoxic tissues. Hyperbaric oxygen (HBO) therapy involves the administration of 100% oxygen at pressures two or thee times greater than normal (sea level) pressure. When the patient breathes 100% oxygen while their whole body is under pressure, greater amounts of oxygen are dissolved into the bloodstream and are delivered to the body tissues in amounts sufficient to promote healing. The use of HBO significantly enhances delivery of oxygen to compromised tissues and thereby restores cellular function, promotes healing, and neovascularisation (Youn 1996).

Preliminary investigations using rested anaesthetised yellow-eye mullet (A. forsteri) held in oxygen saturated seawater under hydrostatic pressure of ~2 atm indicated that fish treated in this way prior to slaughter had higher WM cut-surface pHs during PM storage than fish that had not had the treatment (unpublished data, Jerrett et al. 1996). The electrical excitability (indicating nerve and muscle cell function) was also maintained for longer in the hyperoxia treated fish. These results were very encouraging, however, a disadvantage of the hydrostatic pressure protocol was that even though the fish were heavily sedated prior to entering the hydrostatic pressure chamber, and there was anaesthetic in the water during the treatment, the fish became agitated as the chamber was pressurised or depressurised. Studies have shown that the effects of anaesthetics on vertebrates can be eliminated by pressures of ~100 atm (Smith et al. 1984). However, in this case the effect was attributed to the effects of high oxygen tension rather than the direct effects of the pressure changes and hydrostatic pressure.

It was initially seen as an advantage to use live fish due to the circulation being intact and efficient uptake of oxygen at the gills and delivery of oxygen to tissues being possible. But because the fish became distressed during the treatment (rested harvesting could not be achieved) this method was abandoned. As using live, whole fish was not an option for the continuation of the research, it was decided that an ischemic preparation (WM fillets) held in dry conditions at the appropriate optimum storage temperature would be trialed. Although the ischemic WM preparation had no circulatory system it was hypothesised that increased amounts of oxygen would be delivered to the tissue by diffusion if held under pressure with 100% oxygen. By flushing the tissue with pure oxygen, this would theoretically increase the rate of diffusion 5-fold in comparison with air. By then placing the WM under pressure (in this

case 620 kPa, ~6 atm) the rate of diffusion was increased 30-fold overall. Unlike the hyperoxic pre-loading experiments using the live fish, this preparation could be continuously exposed to the treatment during storage.

The objective of this study was to determine the effects of hyperbaric oxygen storage on the PM metabolism of rested, ischemic mullet WM. The effects were compared with the PM metabolism of ischemic WM stored under normobaric conditions with oxygen flow (characterised in the previous chapter). Storage of the WM under pressure at the appropriate temperature reduced the rate of pH decline by two thirds of the rate exhibited by the normobaric control. The onset of ATP and glycogen depletion, with its concomitant increases in tissue lactate, P_i and creatine, was delayed by ~27 h. However, even with the increased oxygen supply, the tissue still showed a reduction in pH, albeit at a much reduced rate. Below tissue pH values of 7.1 aerobic respiration was either inhibited or the rate of ATP generation was insufficient to cope with demand and anaerobic glycolytic generation of ATP was switched on.

4.3 MATERIALS AND METHODS

4.3.1 Experimental timing and acclimation temperature

Yellow-eye mullet captured in 1996 and 1998 were used in the following experiments:

Four hyperbaric treatments were tested at two sampling times in December 1997 and January 1998 in order to determine the storage pressure that retarded PM metabolic acidosis in the WM the most. The mean seawater temperature for the 40 d period spanning the December 1997 and January 1998 samples was 18.8 ± 0.2 °C (± SEM).

Mullet sampled in February 1998 were used to determine the effect of oxygen flow through the storage chamber on PM metabolism of the rested WM. The mean seawater temperature for the 14 d period prior to the February 1998 sample was 21.6 ± 0.3 °C (± SEM).

Experiments were carried out in January and February 1998 to determine how storage temperature affects the PM metabolism of the rested WM. The mean seawater temperature for the 23 d period spanning the January and February 1998 samples was 21.5 ± 0.2 °C (± SEM).

After determining the PM storage pressure that was most beneficial for the rested WM, an experiment was carried out in December 1998 to further characterise the PM metabolism of the WM. The mean seawater temperature experienced by the fish in the 14 d prior to the December 1998 sample was 18.7 ± 0.3 °C (± SEM).

4.3.2 Fish capture protocol

Rested harvesting

Excluding exercised fish (see below), mullet used in the following experiments were harvested using rested harvesting techniques. Tank-rested yellow-eye mullet were anaesthetised with AQUI-S™ Plus at a concentration of 30.0 ± 0.5 mg/L (see Chapter 2: Harvesting Method and Chapter 3: Rested harvesting). The fish were sedated after 5 min and were not suitable for handling until ~25 min. At this point 5 fish were transferred into a 20 L container with 10 L of seawater, containing the appropriate concentration of anaesthetic and transferred to the laboratory. Once in the anaesthetised

state (insensitive to cardiac puncture, ~70 to 80 min exposure), experimentation could begin. Experimental timing was recorded from the introduction of the anaesthetic and the timing of individual measurements were recorded to the nearest minute.

Once fish were anaesthetised a mixed venous blood sample was taken and the pH measured along with lactate and glucose (see Chapter 2: "Blood sampling and pH measurement").

4.3.3 Post-mortem storage of white muscle

The fish were pithed immediately after the blood sample was taken. The fillets were excised from the carcass and the right-hand-side fillets and the left-hand-side fillets of the rested mullet were placed on separate plastic, open mesh racks. Each rack of fillets was placed in a chamber with inlet and outlet pipes for oxygen. Each chamber was then submerged horizontally in a water bath. The specific storage temperature, pressure and oxygen flow rates are described for each specific experiment.

Storage pressure was confirmed with a pressure gauge (Model: DIN 16007, 0-2500 kPa, WIKA Alexander Wiegand GmbH & Co., Klingenberg, Germany) calibrated against a pressure transducer (Model: 422H2-04, Series 422 100 mV, Barksdale Inc., Los Angeles, CA, USA).

Storage temperature was controlled by the refrigerated waterbath (Model: LTD20G, Grant Instruments Ltd., Cambridge, England) and its accuracy confirmed using a Precision Thermometer (Model: 4600, Yellow Springs Instruments Inc., Yellow Springs, Ohio). Gas flows were regulated using a flow meter (Model: C1D-PC, Platon Instrumentation, Basingstoke, England) calibrated against a Mass Flo® Controller (Model: Type 1179A, MKS Instruments, Andover, MA, USA).

4.3.4 Effect of post-mortem storage pressure on post-mortem white muscle metabolism

Four hyperbaric treatments were tested at two sampling times in December 1997 and January 1998 in order to determine which storage pressure was most effective at delaying the loss of ATP and slowing PM acidification in the WM.

In December 1997 (ambient water temperature = 18.1 ± 0.1 °C) the LHS fillets were pressurised to 260 ± 10 kPa (hyperbaric, hyperoxic; 100% oxygen flow at 50 mL/min ± 1.25%) and the RHS fillet chamber was flushed with air at ambient pressure (normobaric, normoxic; air flow at 50 mL/min ± 1.25%). In January 1998 (ambient water temperature = 20.8 ± 0.1 °C) the LHS fillets were pressurised to 620 ± 10 kPa (hyperbaric, hyperoxic; 100% oxygen flow at 50 mL/min ± 1.25%) and the RHS fillet chamber flushed with 100% oxygen at ambient pressure (normobaric, hyperoxic; oxygen flow at 50 mL/min ± 1.25%).

The higher pressure setting (620 ± 10 kPa) was chosen as this was within the safe working pressures of the plastic pressure housings and was also at the limit of the two-stage regulator available at that time.

Fillet chambers were flushed with the appropriate gas (either humidified air or humidified pure oxygen - 95% humidity) for ~5 min and then the chamber was pressurised to either 260 ± 10 kPa or 620 ± 10 kPa with oxygen flow set to 50 mL/min ± 1.25%. As described in Chapter 3, the flow-rate through the storage chambers was chosen as 50 mL/min as preliminary investigations using flow-rates of both 50 and 500 mL/min did not result in any significant difference in WM acidification rates during PM normobaric or hyperbaric storage.

The chambers were submerged horizontally in a water bath at 10.0 ± 0.1 °C. The cut-surface pH of the WM was measured immediately after filleting and then 10 and 25 h after introduction of the anaesthetic for fillets held at 260 ± 10 kPa (oxygen flow 50 mL/min ± 1.25%) and normobaric pressure (air flow 50 mL/min ± 1.25%), and also at ~37 and ~47 h for fillets held at 620 ± 10 kPa (oxygen flow at 50 mL/min ± 1.25%) and normobaric pressure (oxygen at flow 50 mL/min ± 1.25%).

4.3.5 Effect of oxygen flow through the storage chamber on post-mortem white muscle metabolism stored under hyperbaric conditions

A trial was run in February 1998 with RHS fillets pressurised to 620 ± 10 kPa with no oxygen flow and LHS fillets held at the same pressure but with oxygen flow set to 50 mL/min ± 1.25%.

Both hyperbaric treatment chambers were submerged in a water bath at 10.0 ± 0.1 °C (ambient water temperature = 22.3 ± 0.1 °C). The cut-surface pH of the WM was measured immediately after filleting and then 12, 27 and 37 h after introduction of the anaesthetic.

4.3.6 Effect of low post-mortem storage temperature on white muscle metabolism stored under hyperbaric conditions

Using the standard hyperbaric storage protocol (620 ± 10 kPa pressure with oxygen flow at 50 mL/min ± 1.25 %), three experiments were carried out during January and February 1998 with ischemic WM stored in a waterbath at either 2.0 ± 0.1, 6.0 ± 0.1 or 10.0 ± 0.1 °C. The ambient water temperatures for the three temperature experiments were 19.1 ± 0.1, 21.0 ± 0.1 and 20.8 ± 0.1 °C respectively. WM pH was measured immediately after harvest and then 12, 27, 37 and 48 h after introduction of the anaesthetic.

4.3.7 Characterisation of the post-mortem metabolism of white muscle stored under hyperbaric conditions

In December 1998 an experiment was carried out to compare the metabolic effects of hyperoxic, normobaric storage with hyperbaric, hyperoxic storage using the methods detailed in Chapter 3, Section 3.1. LHS fillets were pressurised to 620 ± 10 kPa with oxygen flow set to 50 mL/min ± 1.25%, and the RHS fillets stored under normobaric conditions with oxygen flow set to 50 mL/min ± 1.25%. Both treatments were submerged in a water bath at 10.0 ± 0.1 °C (ambient water temperature = 20.8 ± 0.1 °C). The cut-surface WM pH measurements and WM samples were taken from the fillets prior to going into storage and then 12, 27, 37, 50, and 57 h after introduction of the anaesthetic. WM samples were taken and freeze-clamped to be analysed for metabolites (lactate, P_i, ATP, creatine and glycogen) at a later date (see Chapter 2 for methods).

4.3.8 Oxygen penetration into the white muscle during post-mortem storage

In similar experiments to those described above an attempt was made to measure the level of oxygen in the hyperbaric, hyperoxic WM during PM storage. This was done by inserting an oxygen electrode (Model: MI-730; Microelectrodes, Inc., Bedford, NH, USA; connected to a dissolved oxygen meter Model: OM-4; Microelectrodes, Inc., Bedford, NH, USA; calibrated as directed) into the anterior cut end of the fillet after a pH measurement was made during PM storage.

4.3.9 Statistical analysis

All times, pH values and biochemical values stated in the text are the mean ± standard error of the mean (SEM). Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows (Version 6.00 Copyright© 1996-2000, SPSS inc.) and Microsoft® Excel 2000.

4.4 RESULTS

4.4.1 Effect of post-mortem storage pressure on white muscle metabolism

Post-harvest condition

Mullet sampled in January 1998 were significantly longer than the fish sampled in December 1997 (Table 4.1). The weight of the December 1997 fish was not measured. Fish sampled in December 1997 had significantly lower ($P < 0.05$) blood pHs than mullet sampled in January 1998 (Table 4.1). A possible reason for this was that the fish sampled in December 1997 may have exercised or been disturbed prior to the anaesthetic being added to the tank, resulting in a slight acidosis of the blood. The increase in metabolic rate of the fish would have resulted in a more rapid uptake of the anaesthetic and hence, an earlier pithing time compared with mullet sampled in January 1998 (Table 4.1). Even though the December 1997 fish may have exercised prior to anaesthesia there was no difference in pre-storage WM pH between the two groups of fish (Table 4.2).

Table 4.1. *Size, blood pH and pre-storage white muscle pH in mullet used to determine the most supportive post-mortem storage pressure.*

	December 1997 Air 50 mL/min & Oxygen 260 kPa50 mL/min (n = 5)	**January 1998** Oxygen 50 mL/min & Oxygen 620 kPa 50 mL/min (n = 5)
Weight (g)	-	117.1 ± 5.8
Length (mm)	191 ± 4	204 ± 4*
Pithing time (h)	1.34 ± 0.03	1.70 ± 0.03*
Blood pH	7.44 ± 0.07	7.61 ± 0.05**

Values are the mean ± SEM, n = 5.
* Significantly different from the December 1997 mullet ($P < 0.05$; two-tailed Student's t-test).
** Significantly different from the December 1997 mullet ($P < 0.05$; one-tailed Student's t-test).

Table 4.2. Pre-storage white muscle sampling time and cut-surface pH measurement of mullet sampled to determine the effect of storage pressure on white muscle metabolism.

	December 1997		January 1998	
Storage details	Air 50 mL/min	Oxygen 260 kPa 50 mL/min	Oxygen 50 mL/min	Oxygen 620 kPa 50 mL/min
Sampling time (h)	1.84 ± 0.08	1.85 ± 0.08	2.10 ± 0.08*	2.13 ± 0.08*
Initial WM pH	7.60 ± 0.04	7.63 ± 0.02	7.61 ± 0.04	7.66 ± 0.03

Values are the mean ± SEM, n = 5.
* Significantly different from the December 1997 mullet ($P < 0.05$; two-tailed Student's t-test).

Post-mortem white muscle pH profiles

The PM pH profiles of mullet WM held at different storage pressures (hyperbaric and normobaric) and different gas mixtures (air or 100% oxygen; normoxic and hyperoxic respectively) are shown in Fig. 4.1. When the fillets were stored under normobaric, hyperoxic conditions (ambient pressure with oxygen flow through the storage chamber) the WM pH after 12 h was significantly higher ($P < 0.05$) than fillets stored under normobaric, normoxic conditions (ambient pressure with air flow through storage chamber). However, after 25 h there was no difference in WM pH between the two normobaric treatments. Increasing the storage pressure to 260 ± 10 kPa with oxygen (hyperbaric, hyperoxic) slowed the PM pH decline in comparison with fillets held under normobaric, hyperoxic conditions. Increasing the storage pressure to 620 ± 10 kPa with oxygen flow produced the slowest rate of pH decline (Fig. 4.1).

Fig. 4.2 shows the effect of increasing oxygen pressure on the time it takes for the PM pH of the WM to reach either pH 7.0 or pH 6.4. Storage at the highest pressure greatly extended the time taken for the pH to fall to 6.4. The time taken for the pH to fall to 7.0 was less affected by increasing pressure. From these results a decision was made to make the standard storage pressure 620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%. The standard control to the hyperbaric, hyperoxic treatment was then taken to be normobaric pressure with humidified oxygen flow set to 50 mL/min ± 1.25% (normobaric, hyperoxic).

4.4.2 Effect of oxygen flow through the storage chamber on post-mortem white muscle metabolism stored under hyperbaric, hyperoxic conditions

The weight, length and condition factor of the fish used in this experiment are shown in Table 4.3.

Table 4.3. *Size, condition factor and hepatosomatic index of mullet used in each experiment.*

Experiment	Weight (g)	Length (mm)	CF[1]	HSI[2]
Gas Flow	100.0 ± 4.1	196 ± 2	1.33 ± 0.04	1.43 ± 0.02
Storage temperature 2°C	124.1 ± 10.9	211 ± 6	1.31 ± 0.04	1.69 ± 0.15
6°C	135.6 ± 10.9	210 ± 5	1.45 ± 0.06	2.03 ± 0.20
10°C	133.8 ± 9.7	213 ± 4	1.37 ± 0.04	1.63 ± 0.28
PM metabolism	214.8 ± 23.2*	247 ± 7*	1.40 ± 0.04	2.93 ± 0.41*

[1]Condition factor (CF) = weight (g)/length (mm^3) x 100000 (Love 1980).
[2]Hepatosomatic index (HSI) = liver weight (g)/weight (g) x 100 was (Love 1980).
Values are the mean ± SEM, n = 5.
* Significantly different from the other experiments ($P < 0.05$; Student's t-test).

Post-harvest condition

The immediate post-harvest, pre-storage cut-surface pH of the D1 WM was measured at a mean time of 1.45 ± 0.08 h in fish that were to be stored under hyperbaric conditions with and without oxygen flow through the storage chambers. There was no significant difference in pre-storage WM pH (Fig. 4.3). The effect of gas flow through the fillet chamber on PM cut-surface pH is shown in Fig. 4.3. The WM pH decline in ischemic WM held under pressure with no oxygen flow was faster than in WM held under pressure with flow. But the PM decline in pH performed only slightly better than WM stored under normobaric conditions with oxygen flow through the storage chamber (data from "Effect of post-mortem storage pressure", see Fig 4.1). Oxygen flow through the storage chamber in conjunction with the standard storage pressure (620 ± 10 kPa) slowed the rate of PM pH decline the most compared with fillets held under the same conditions with no oxygen flow.

4.4.3 Effect of low post-mortem storage temperature on white muscle metabolism stored under hyperbaric, hyperoxic conditions

There was no significant difference between weight, length, CF and HSI of fish stored at either 2.0, 6.0 and 10.0 °C (Table 4.3). The initial WM cut surface pH was measured in the 2.0 °C trial at 1.25 ± 0.12 h, 6.0 °C at 1.36 ± 0.13 h and 10.0 °C at 1.34 ± 0.03 h after the introduction of anaesthetic. There was no significant difference in initial WM pH between the three groups of fish (Fig. 4.4). There was no difference in PM cut-surface pH decline in WM that was stored under the standard protocol (620 ± 10 kPa, oxygen flow of 50 mL/min ± 1.25%) at 2.0, 6.0 or 10.0 °C (Fig. 4.4).

4.4.4 Characterisation of the metabolism of white muscle stored under hyperbaric, hyperoxic conditions

Post-harvest condition

The fish used in the experiment were the same fish as those used in Chapter 3 (rested summer 1998 fish). The LHS fillets of those fish acted or were used as the control for the hyperbaric, hyperoxic treatment. Blood was sampled from the rested fish at a mean time of 0.61 ± 0.03 h after introduction of the anaesthetic. The mean blood pH was 7.69 ± 0.04, blood lactate 1.85 ± 0.60 mmol/L and blood glucose 6.09 ± 0.28 mmol/L. The immediate post-harvest, pre-storage cut-surface pH of the D1 WM in the rested fish was measured at a mean time of 1.13 ± 0.12 h and was 7.61 ± 0.02. The mean pre-storage metabolite levels were as follows: lactate = 12.7 ± 1.0 μmol/g; ATP = 7.4 ± 0.7 μmol/g; P_i = 22.9 ± 2.1 μmol/g; creatine = 12.6 ± 1.0 μmol/g; glycogen = 37.6 ± 5.6 μmol/g.

Post-mortem white muscle pH profiles

The WM pH profiles of fillets held under hyperbaric or normobaric conditions are shown in Fig. 4.5. In the normobaric treatment the WM pH dropped rapidly from 1 to 27 h and had reached the ultimate pH (~6.40) after 27 h. In hyperbaric fillets the WM pH rate of decline was much slower over the 56 h storage period, reaching the ultimate pH of 6.4 at 56 h.

Oxygen penetration into the white muscle

During the sampling process it was also noted that bubbles could be observed in the tissue when looking at the cut-surface. These could be seen after 27 h storage. Up until 11 h storage no oxygen could be detected in the WM when an oxygen electrode was inserted into the tissue. After 27 h storage high levels of oxygen were measured suggesting that the bubbles observed were, in fact, oxygen. There was a possible error with this method in that the probe may have separated muscle blocks as it was being inserted into the WM and was only measuring the bubbles formed from depressurisation of the WM. Therefore, it was unclear as to whether accurate oxygen levels were being measured.

Post-mortem white muscle metabolite profiles

Under normobaric conditions [lactate] increased rapidly over the first 27 h of storage reaching a peak of ~70 μmol/g muscle mass (Fig. 4.6a). In hyperbaric tissue a 12 h delay in the rise of [lactate] was observed. Lactate then continued to accumulate and peaked at ~75 μmol/g muscle mass after 57 h storage (Fig. 4.6a). The relationship between WM pH and WM [lactate] is shown in Figure 4.7a and was found to be linear for WM held under both normobaric and hyperbaric conditions. Converting the WM pH into $[H^+]$ showed that there was an exponential relationship between H^+ and lactate concentration in the WM (Fig. 4.7b) of both storage treatments.

Mullet WM held under normobaric conditions showed a rapid depletion in ATP and had reached levels of < 1 μmol/g after 27 h storage (Fig. 4.6b). In the hyperbaric tissue, a delay of ~27 h was seen in the depletion of ATP with levels reaching < 1 μmol/g muscle mass after 50 h storage.

The relationship between pH and ATP is shown in Fig 4.8a. The pH/lactate relationship was stronger than the pH/ATP relationship illustrated by the lower r^2 values for ATP (see Figs. 4.7 and 4.8 legends). When the WM pH was converted to $[H^+]$ there was a distinct biphasic relationship with WM ATP concentration (Fig. 4.8b) in both hyperbaric and normobaric treatments. When the ATP level in the WM was >3 μmol/g muscle mass acidification was minimal. Below 3 μmol/g muscle mass the $[H^+]$ was high.

There was a negative linear relationship between WM lactate and ATP concentration in hyperbaric and normobaric treatment (Fig. 4.9). At high ATP concentrations (start of PM storage) lactate levels were low and conversely, as ATP was depleted lactate levels rose.

A delay in accumulation of P_i of ~12 h was observed in the normobaric WM (Fig. 4.6c). After 12 h storage [P_i] rose steadily and peaked at ~40-45 μmol/g muscle mass after 37 h. Hyperbaric WM did not begin to accumulate P_i until after 27 h storage and then rose to a maximum of ~45 μmol/g muscle mass after 50 h (Fig. 4.6c). As described in Chapter 3 as WM P_i concentration increased WM pH decreased during storage (Fig. 4.10a). The relationship was linear in hyperbaric WM but in normobaric tissue [P_i] continued to increase once the WM had reached its ultimate pH (~6.4). On conversion of the WM pH to [H^+] there appeared to be a step change in acidification occurring around a [P_i] of 35 μmol/g muscle mass (Fig. 4.10b) in both hyperbaric and normobaric WM. Below this [P_i] the WM [H^+] was low, and higher 35 μmol/g muscle mass the majority of [H^+] measurements were between ~3 and 4.5 x 10^{-7} mol/L.

Accumulation of creatine in mullet WM followed a similar pattern to that seen with lactate (Fig 4.6d). There was a rapid rise to a maximum (~22-24 μmol/g muscle mass) after 27 h in normobaric tissue, with a ~12-27 h delay in accumulation of creatine in hyperbaric preparations, followed by an increase peaking at a the same level as the normobaric WM after 37-50 h (Fig. 4.6d).

Although many of the creatine measurements were higher than 20 μmol/g muscle mass and cannot be reliably assessed, the initial rested values were all below this level and were therefore accurate.

Mullet WM glycogen levels (expressed as glucosyl units) are shown in Fig. 4.6e. Prior to storage the rested WM contained ~35-40 μmol/g muscle mass of glycogen. In the normobaric preparation after 37 h storage levels were lower than, and significantly different from initial, pre-storage values. After 37 h storage [glycogen] remained at ~15 μmol/g muscle mass. A delay of 27 h was seen in hyperbaric WM before glycogen started to fall, reaching levels similar to the normobaric values at 50 h. Rates of glycogen depletion were not calculated due to the high level of error in the measurement.

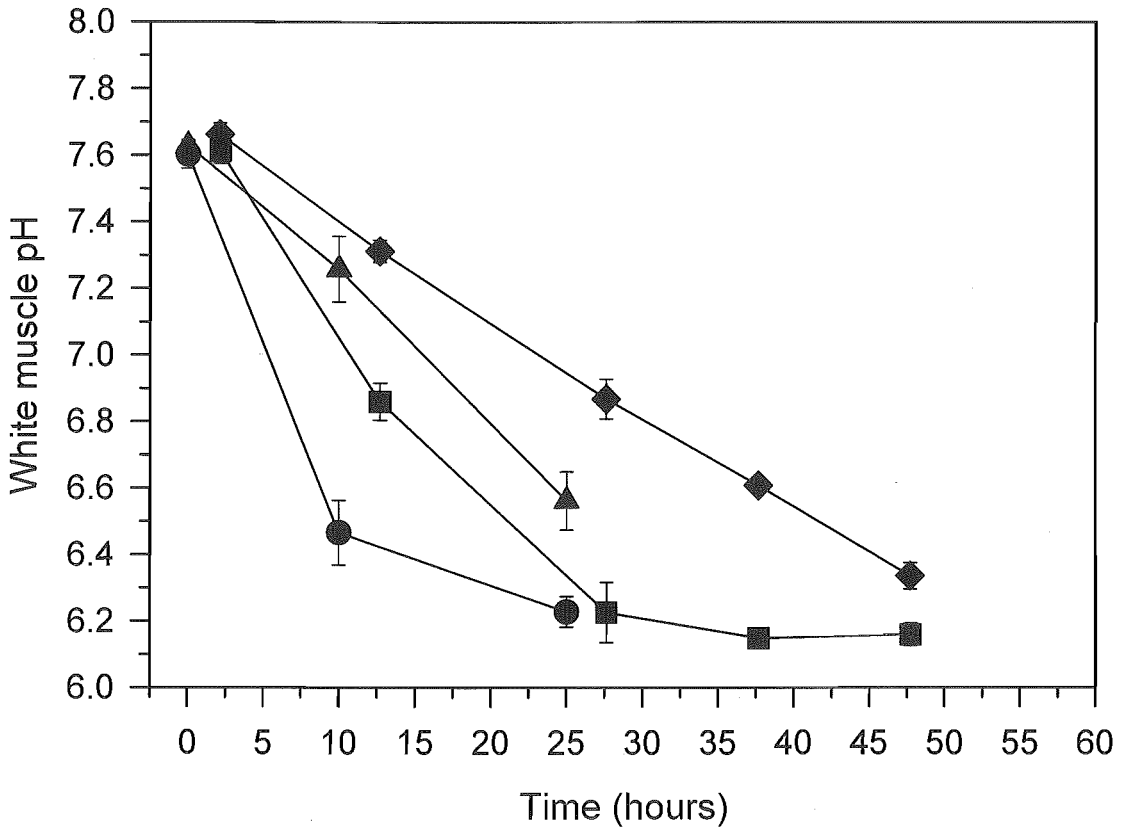

Figure 4.1: *Post-mortem cut-surface pH of the epaxial white muscle from rested yellow-eye mullet stored under normobaric, normoxic conditions (ambient pressure, air-flow 50 mL/min ± 1.25%, ●), normobaric, hyperoxic conditions (ambient pressure, oxygen flow 50 mL/min ± 1.25%, ■), hyperbaric, hyperoxic conditions (260 kPa ± 10 kPa, oxygen flow 50 mL/min ± 1.25%, ▲), and hyperbaric, hyperoxic conditions (620 ± 10 kPa, oxygen flow 50 mL/min ± 1.25%, ♦) at 10.0 ± 0.1 °C. Values are the mean ± SEM; n = 5.*

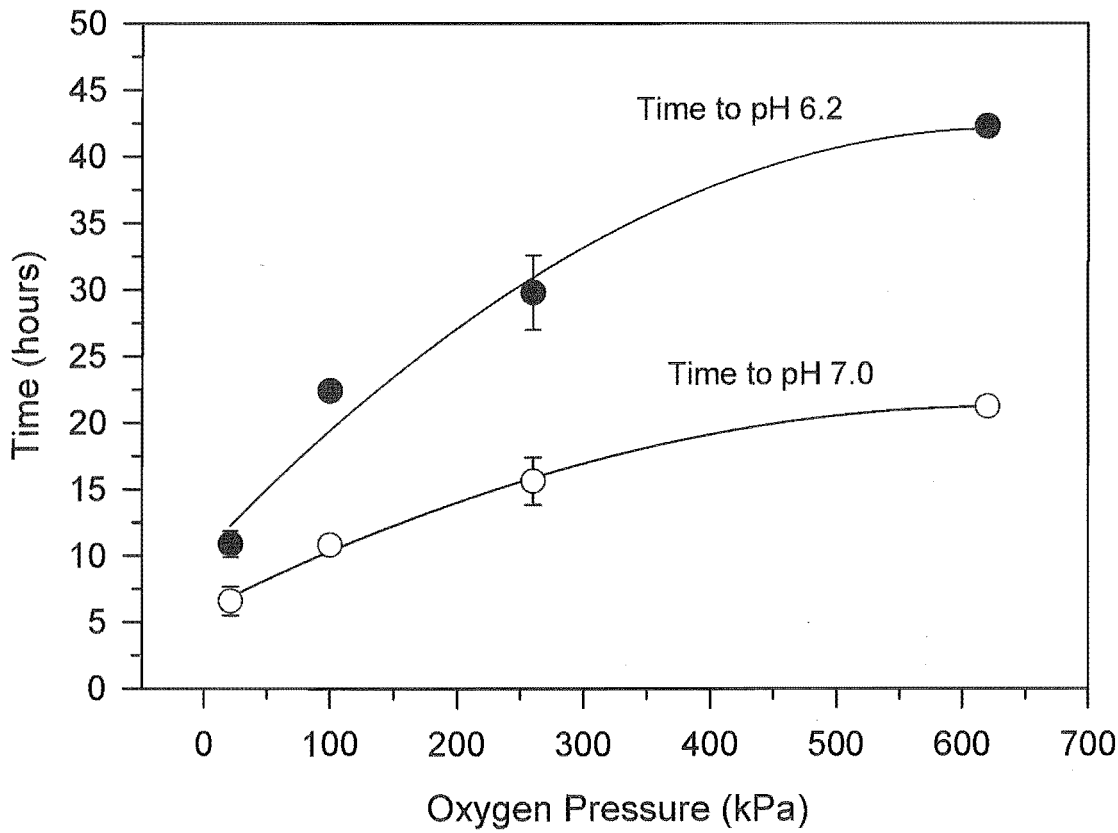

Figure 4.2: *Effect of oxygen storage pressure on the time it takes the cut-surface pH of the epaxial white muscle to reach pH 6.2 (●), or pH 7.0 (○). Values are the mean ± SEM, n = 5.*

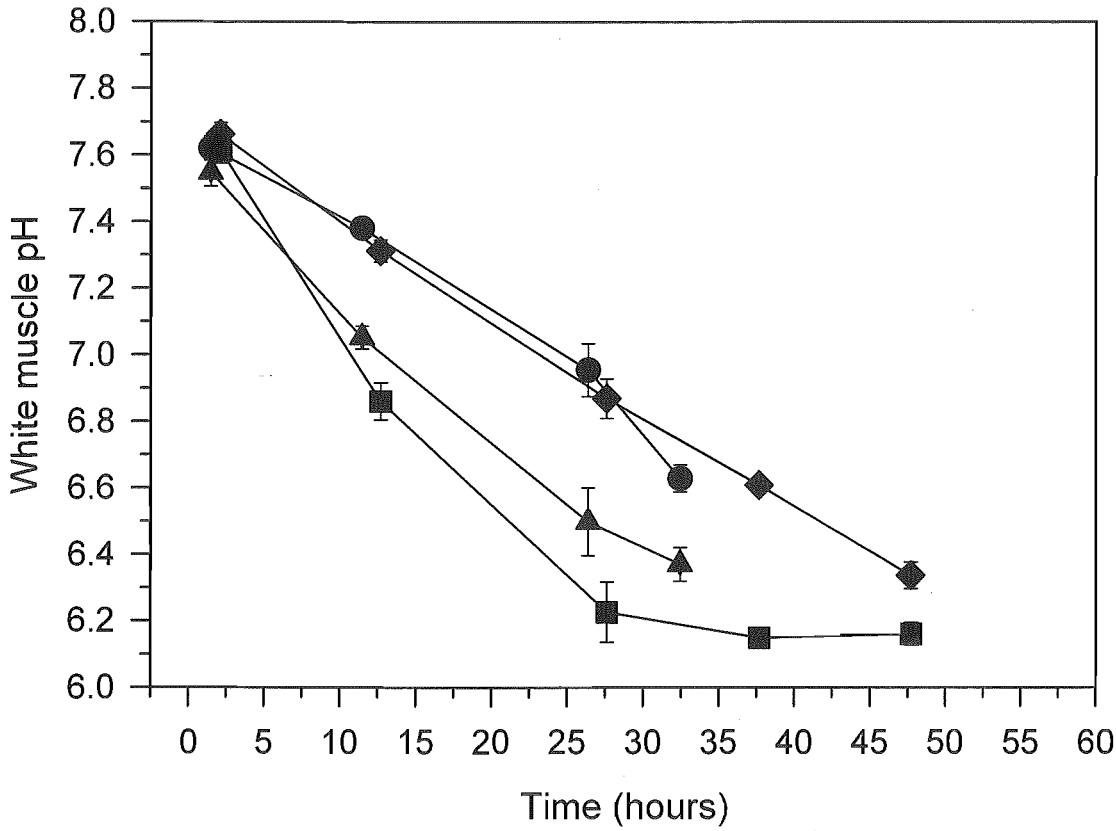

Figure 4.3: *Post-mortem cut-surface pH of the epaxial white muscle from rested yellow-eye mullet stored under hyperbaric (620 ± 10 kPa) hyperoxic conditions with no flow through the storage chamber (▲) and hyperbaric (620 ± 10 kPa) hyperoxic conditions with oxygen flow through the storage chamber (50 mL/min ± 1.25%, ●), compared with data from Fig. 4.1, normobaric, hyperoxic conditions (normobaric pressure with oxygen flow at 50 mL/min ± 1.25% ■) and hyperbaric, hyperoxic conditions (620 ± 10 kPa with oxygen flow at 50 mL/min ± 1.25%, ♦) at 10.0 ± 0.1 °C. Values are the mean ± SEM; n = 5.*

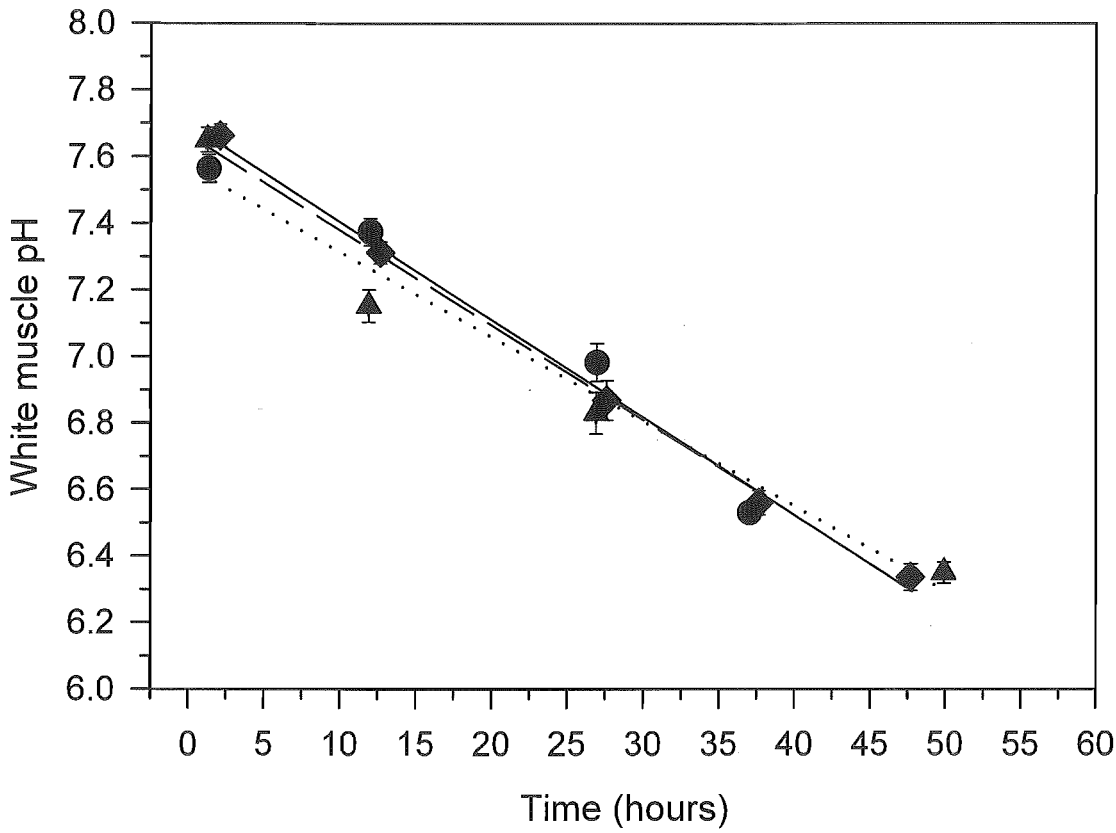

Figure 4.4: *Post-mortem cut-surface pH of the epaxial white muscle from rested yellow-eye mullet stored under hyperbaric, hyperoxic conditions at 10.0 ± 0.1 °C (620 ± 10 kPa with oxygen flow at 50 mL/min ± 1.25%, ♦), 6.0 ± 0.1 °C (●), and 2.0 ± 0.1 °C (▲). Decline in pH with time is described by a linear regression equation: 10.0 °C (solid line) y = -0.029x + 7.70 r^2 = 0.97; 6.0 °C (dashed line) y = -0.028x + 7.66 r^2 = 0.92; 2.0 °C (dotted line) y = -0.025x + 7.57 r^2 = 0.93. Values are the mean ± SEM; n = 5.*

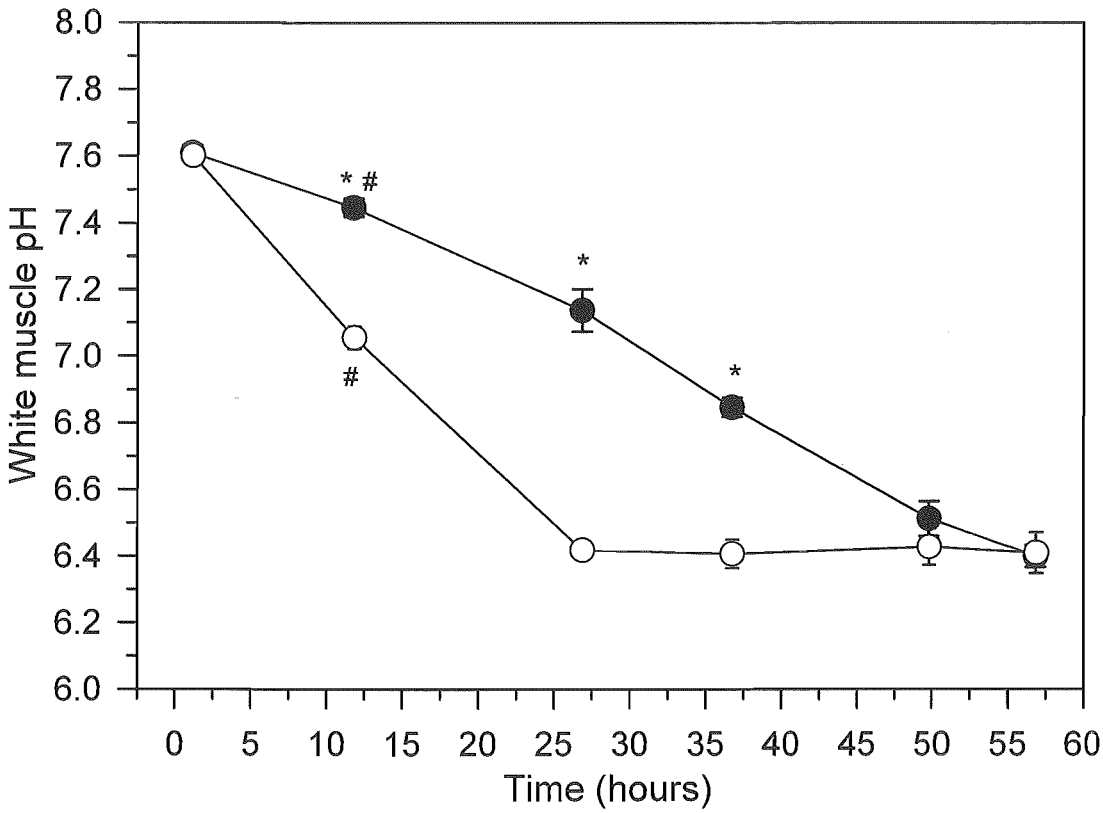

Figure 4.5: *Post-mortem cut-surface pH of the epaxial white muscle from rested yellow-eye mullet stored under normobaric, hyperoxic conditions (normobaric pressure with oxygen flow at 50 mL/min ± 1.25%, ○) and hyperbaric, hyperoxic conditions (620 ± 10 kPa with oxygen flow at 50 mL/min ± 1.25%, ●) stored at 10.0 ± 0.1 °C. Values are the mean ± SEM; n = 5. * Significantly different from the corresponding normobaric value at the same sampling time (Sign test; P < 0.05). # significantly different from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

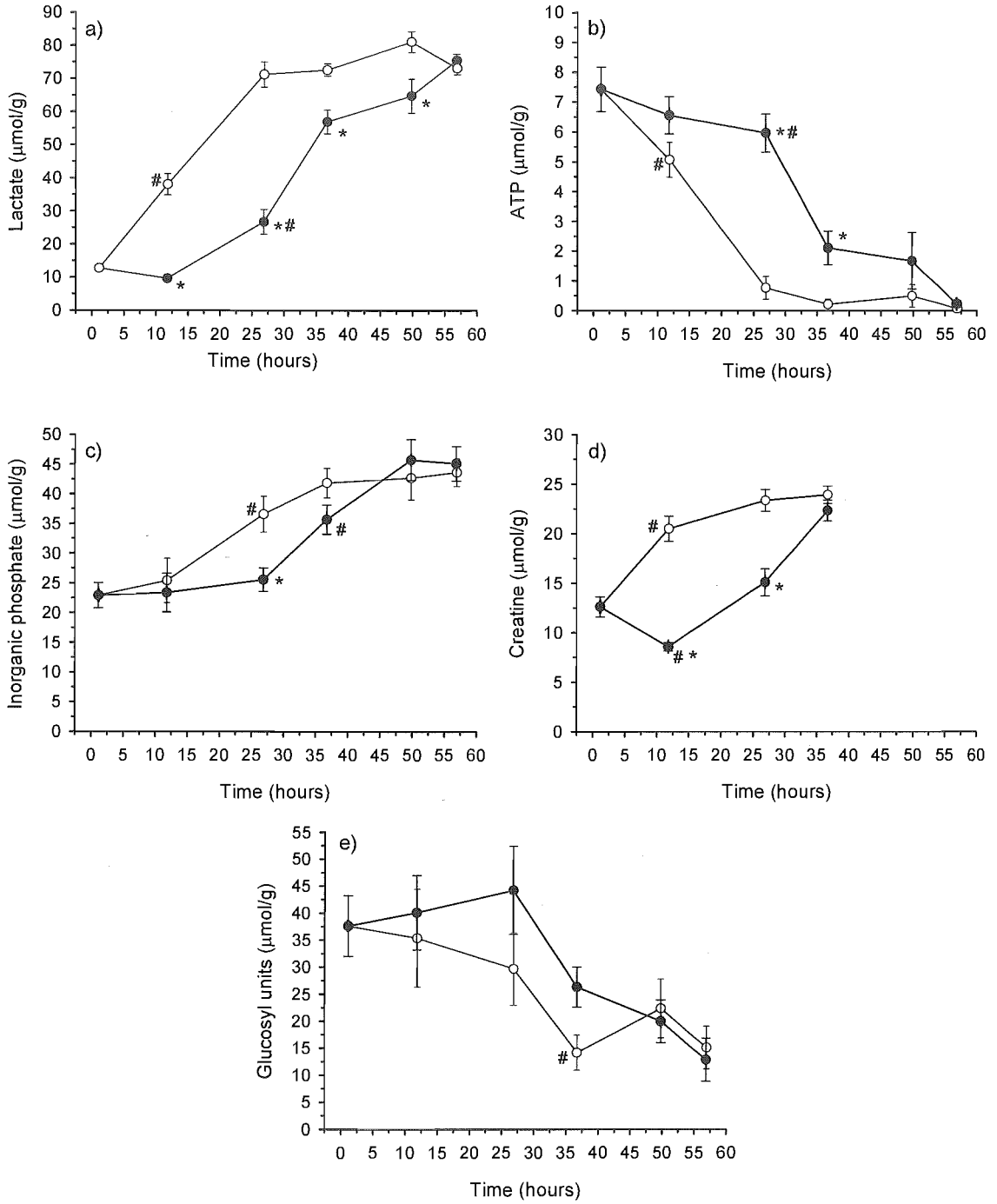

Figure 4.6: *Post-mortem changes in a) lactate, b) ATP, c) P_i, d) creatine and e) glycogen (as glucosyl units) concentration in the epaxial white muscle of rested yellow-eye mullet stored under normobaric, hyperoxic conditions (○) or hyperbaric, hyperoxic conditions (●). For storage details see Fig. 4.6 legend. Values are the mean ± SEM; n = 5. * Significantly different from the corresponding normobaric value (Sign test; P < 0.05). # Significantly different from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

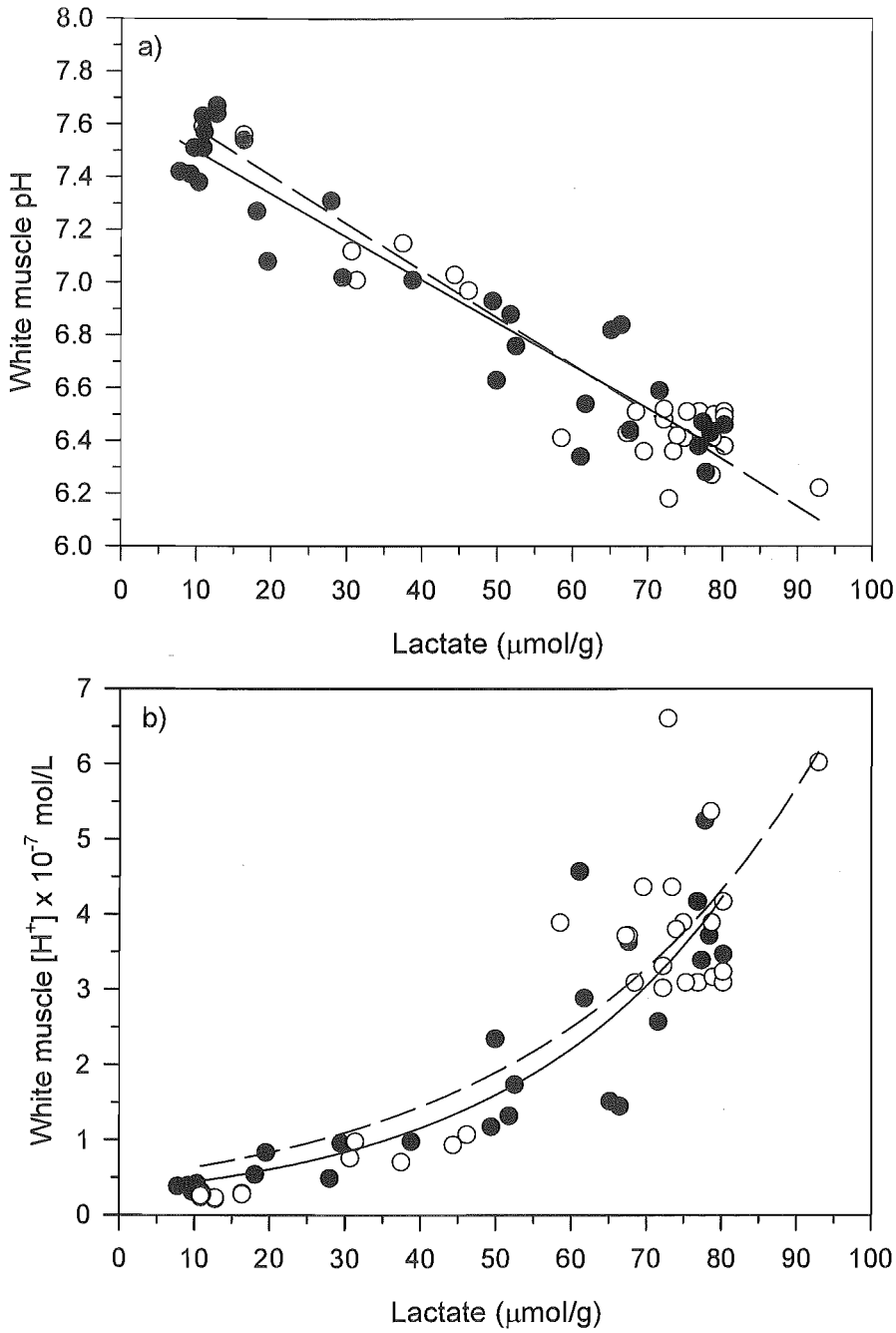

Figure 4.7: *Relationship between a) post-mortem cut-surface pH and [lactate] and b) post-mortem H^+ concentration and [lactate] in the epaxial white muscle from summer (1998) rested yellow-eye mullet stored under hyperbaric (●) or normobaric (○) conditions. The pH/[lactate] relationship for hyperbaric white muscle (●, solid line) is described by the linear regression equation* y = *-0.016x + 7.66, r^2 = 0.90 and for normobaric white muscle (○, dashed line) y = -0.018x + 7.76, r^2 = 0.93. The regression equations were not significantly different (Student's t-test). The $[H^+]$/[lactate] relationship for hyperbaric white muscle is described by the exponential equation $y = 0.32e^{0.028x}$, $r^2 = 0.79$ and in normobaric white muscle $y = 0.48e^{0.028x}$, $r^2 = 0.75$.*

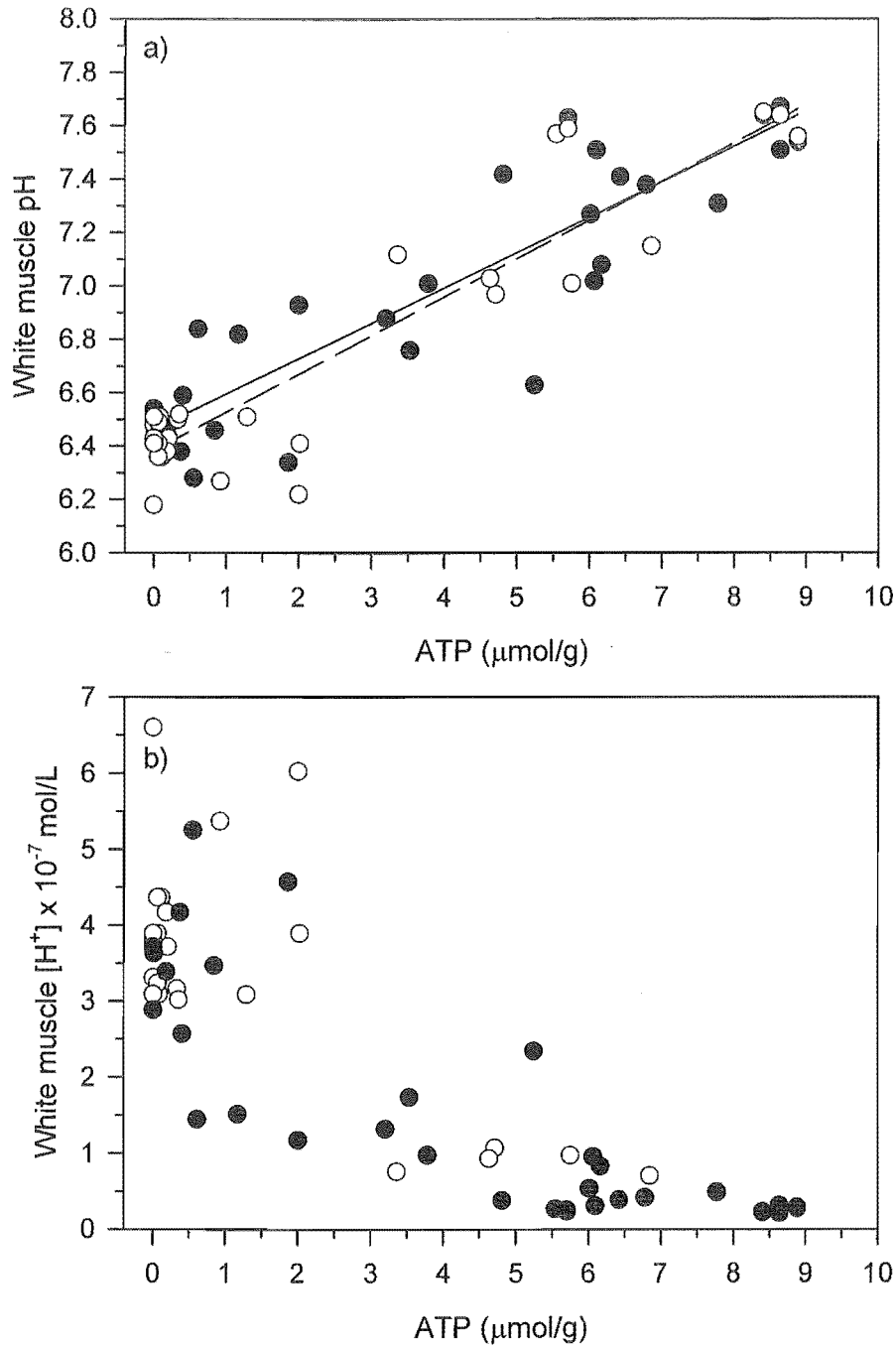

Figure 4.8: *Relationship between a) post-mortem cut-surface pH and [ATP] and b) post-mortem hydrogen ion concentration and [ATP] in the epaxial white muscle from summer (1998) rested yellow-eye mullet stored under hyperbaric (●) or normobaric (○) conditions. The pH/[ATP] relationship for hyperbaric white muscle (●, solid line) is described by the linear regression equation* y = 0.133x + 6.46, r^2 = 0.78 *and for normobaric white muscle (○, dashed line)* $y = 0.144x + 6.38$, $r^2 = 0.86$. *The regression equations were not significantly different (Student's t-test). No curves were fitted for the* $[H^+]$/[ATP] *relationship.*

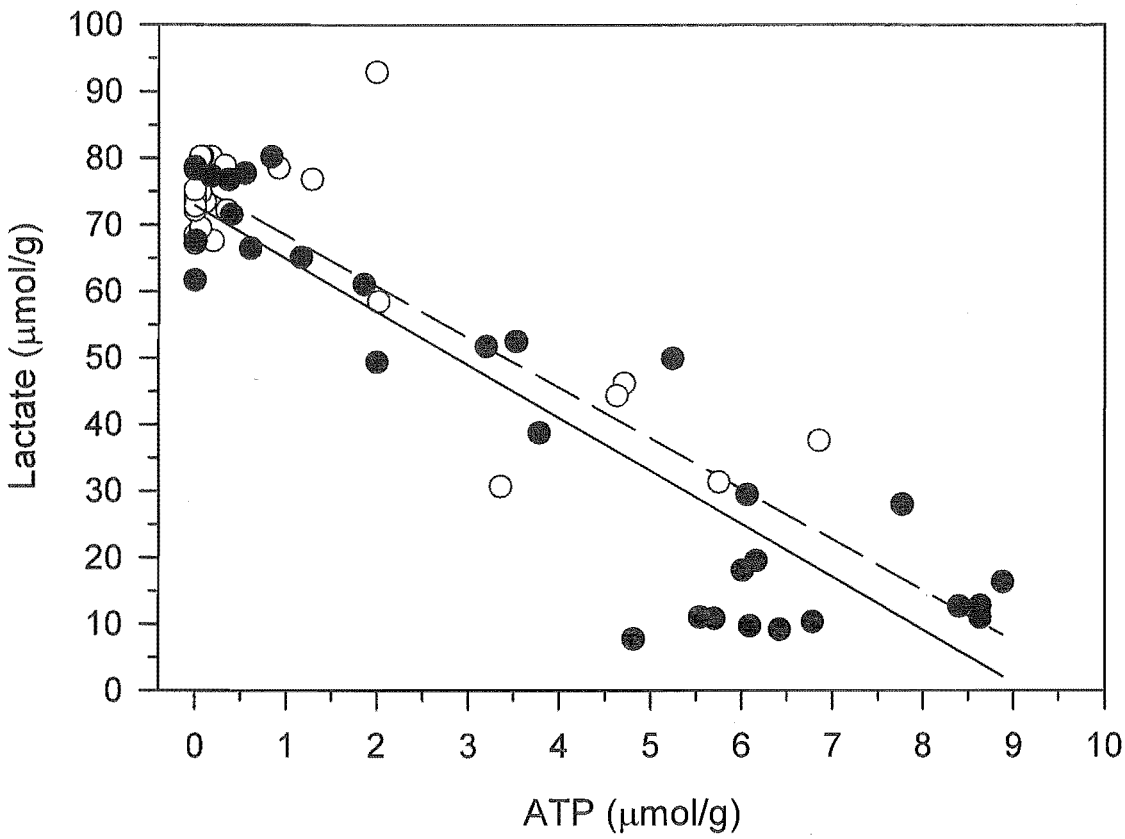

Figure 4.9: *Relationship between post-mortem white muscle lactate and ATP concentration in the epaxial white muscle from summer (1998) acclimated rested yellow-eye mullet stored under hyperbaric (●) or normobaric (○) conditions. The relationship in hyperbaric white muscle is described by the linear regression equation:* $y = -7.97x + 72.86$, $r^2 = 0.83$ *(solid line) and in normobaric white muscle* $y = -7.94x + 76.21$, $r^2 = 0.83$ *(dashed line).*

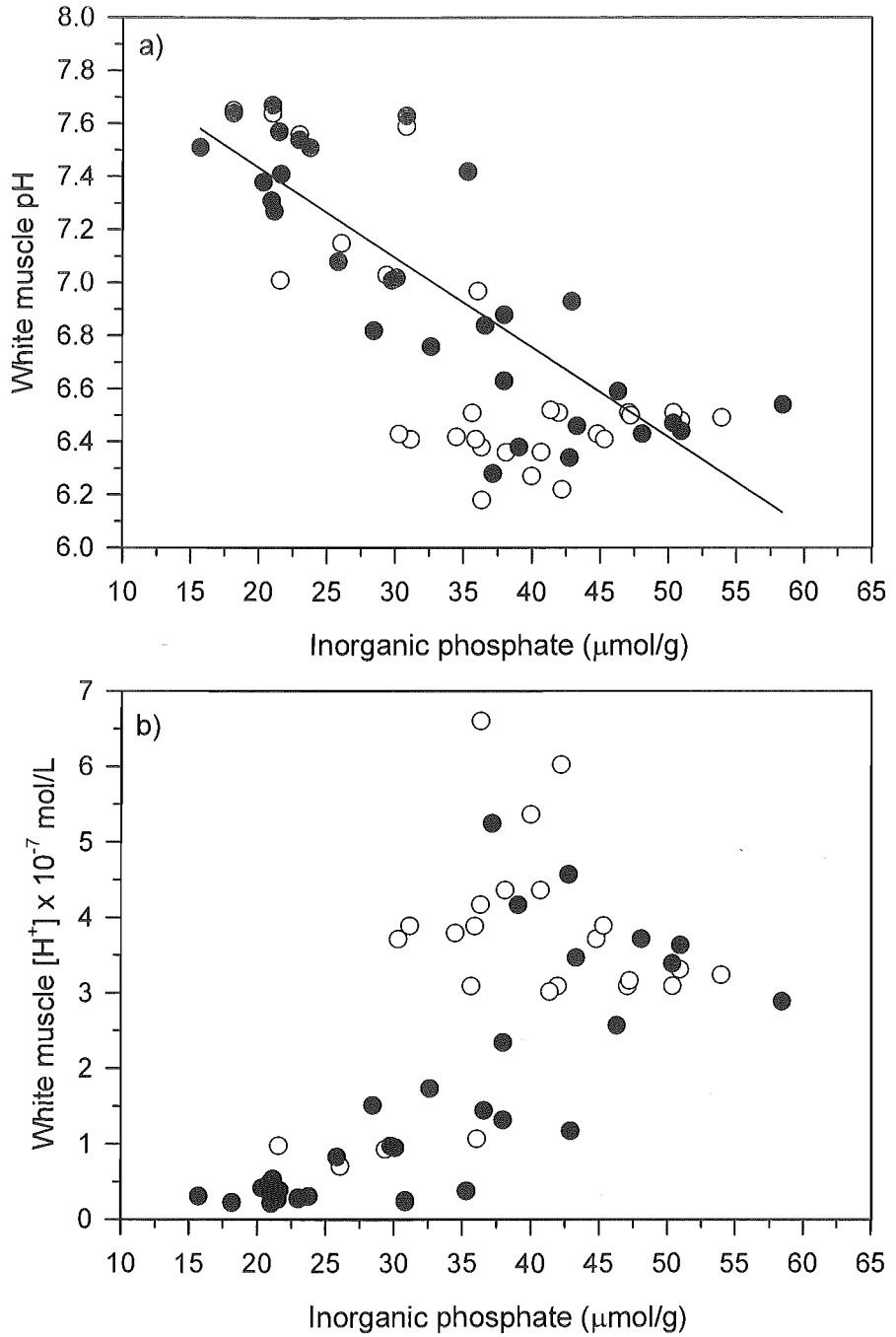

Figure 4.10: *Relationship between a) post-mortem cut-surface pH and [P_i] and b) post-mortem H^+ concentration and [P_i] in the epaxial white muscle from summer (1998) rested yellow-eye mullet stored under hyperbaric (●) or normobaric (○) conditions. The pH/[P_i] relationship for hyperbaric WM (●, solid line) is described by the linear regression equation* $y = -0.034x + 8.11$, $r^2 = 0.67$. *No curves were fitted to the other data. The correlation coefficient for the hyperbaric pH/[P_i] relationship is –0.83 and in normobaric WM -0.73. For the [H^+]/[P_i] relationship the correlation coefficient for hyperbaric WM is 0.76 and in normobaric WM is 0.61.*

4.5 DISCUSSION

4.5.1 Methodology

Measuring the cut-surface pH of the WM in the same fillets throughout the storage period meant that the hyperbaric tissue had to be depressurised at each measurement time. This may have had a positive effect on the maintenance of the tissue by "washing-out" waste products of metabolism (i.e. CO_2) and could have altered the WM metabolism. However, in a preliminary investigation to determine if depressurisation after 10 h had any effect (positive or negative) on the PM metabolism of the WM, ischemic WM was stored using the standard hyperbaric storage protocol but was not disturbed until after 25 h storage. There was no significant difference in the rate of acidification in the WM between fillets that had been depressurised after 10 h or those that had only been depressurised after 25 h (results not presented here). Therefore any effect of depressurisation on the WM was thought to be minimal.

When the fillets were taken out of the storage chamber to have the WM pH measured they would have increased in temperature from the storage temperature, possibly affecting the rate of metabolism for a short time. This may have resulted in a slight hastening of metabolic changes during PM storage. The WM cooling rate could have been better characterised as the excised tissue was stored in air (not RSW as other studies have done) and therefore the rate of cooling would have been much slower, but, irrespective of the absolute cooling rates normobaric and hyperbaric tissues were exposed to a similar cooling regime and were estimated to reach the set temperature within an hour.

Oxygen penetration into the white muscle

It was assumed that the oxygen was, in fact, penetrating the WM during PM storage under hyperbaric, hyperoxic as after ~27 h storage small bubbles were observed in the WM and oxygen could be measured in the muscle (by inserting an oxygen probe) when pH measurements were made. As mentioned in the Results section there were possible errors with this method. Further attempts to accurately measure the oxygen content of the WM were not pursued as the focus of the study was modification of the PM metabolism in WM that was not compromised by dissection artefacts. Inserting a

probe into the fillet damaged the tissue and would have inevitably affected the metabolism. It was also uncertain if the membrane of the oxygen probe could tolerate such treatment, and if it did, what was the probe measuring. Chemical methods for determining oxygen content in small pieces of tissue were also investigated. Because bubbles (presumably oxygen) formed in the WM upon depressurisation it was thought that any measurement would be an over-estimate of the level under hyperbaric conditions. Also, dissection of the small pieces of WM required for the measurement would have further introduced artefacts into the measurement.

The physical and safety limitations of the experimental set-up for PM storage under hyperbaric, hyperoxic conditions meant that there was a limit to the pressure the WM could be stored under (in this case 620 kPa). Because there was found to be a significant difference in the rate of WM acidification when tissue was stored at that pressure a decision was made to continue the investigations using this pressure instead of designing an entirely new experimental set-up that would allow testing of higher pressures. This will be discussed in the following section.

Measurement of the WM pH during PM storage gave a fast and repeatable measure of the physiological status of the WM. From the results it could be shown that WM pH was indicative of both lactate and ATP levels in the WM (Figs. 4.8 & 4.9). It did not give a good estimate of the metabolic rate of the WM unless the WM was under hypoxic conditions (i.e. normobaric conditions) when it respired anaerobically and the acidification rate was directly related to production of lactate. Under hyperbaric, hyperoxic conditions the WM acidification rate was only indicative of the metabolic rate and could indicate the extension of cell viability.

4.5.2 Effect of post-mortem storage pressure on white muscle metabolism

Storing ischemic WM under hyperbaric, hyperoxic conditions had a significant negative effect on the rate of PM metabolic acidosis as measured by cut-surface pH (Fig. 4.1 & 4.2). This most likely indicated supplementation of the ATP supply by oxidative phosphorylation. This was further supported by the observation that flushing the chamber produced lower rates of acidification (Fig. 4.3) compared with hyperbaric storage with no oxygen flow.

In this study there was a limit to the level of pressure that the ischemic WM could be stored under due to limits of the equipment available. However, it would have been of interest to investigate the effects of higher pressures to determine if the WM tissue acidification could be slowed even further or if there was a point where the rate was accelerated. In a preliminary investigation (data not presented here) different experimental apparatus was used in order to test the effects of a higher storage pressure (1220 kPa). It was not possible to have oxygen flowing through the fillet chamber with this apparatus at this pressure but the chamber volume was considerably larger. It was thought that any change to the rate of acidification would be obvious after the first 10 h of storage. However, there was no significant difference in tissue pH compared with the standard hyperbaric, hyperoxic storage protocol (620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%). After 27 h storage the WM pH had decreased to a lower level compared with the standard storage protocol, perhaps indicating a combination of metabolic and "respiratory" acidosis. It was possible that beyond a certain storage pressure it was the clearance of CO_2 that became more important than the supply of oxygen to the WM. This will be discussed in the following section. The data plotted in Fig 4.2 may tentatively support the conclusion that oxygen pressures higher than ~620 kPa may have done little to alleviate tissue acidification either by meeting the oxygen demand or perhaps increasing tissue stress.

4.5.3 Importance of oxygen flow during post-mortem storage

In the current study, WM held under hyperbaric, hyperoxic conditions with no flow through the storage chamber had a similar PM rate of WM acidification as WM held under normobaric, hyperoxic conditions (Fig. 4.3). When the WM was stored under the standard hyperbaric, hyperoxic conditions, it was assumed that the reduction in the rate of WM acidification during PM storage was due to ATP being generated via oxidative phosphorylation rather than anaerobic glycolysis. By definition, the ischemic preparation has no blood supply, preventing the elimination of waste products into the circulatory system that may be harmful to the cell. One waste product that has received little attention in the general literature is CO_2. This may be due to the fact that most studies are focused on teleost exercise and recovery metabolism. Acidification of WM by end products of respiration such as CO_2 is difficult to measure and in constant, rapid flux as the circulation washes them out during recovery.

CO_2 production occurs during oxidative phosphorylation when pyruvate is oxidised to acetyl CoA (2 molecules of CO_2 produced) and in the Citric Acid Cycle (4 CO_2's produced). Thus for every glucose molecule oxidised, 6 CO_2's are produced (Mathews & van Holde 1990).

Respiratory acidosis would not normally be significant in teleost WM because of its hypoxic nature. Gas flow through the storage chamber may have assisted diffusion of CO_2 out of the tissue. Given the high solubility of CO_2, without a circulatory system, or the enhanced diffusion gradient provided by the gas flow, the CO_2 may have hastened the acidification of the WM or potentially inhibited oxidative phosphorylation.

4.5.4 Effect of storage temperature on post-mortem metabolism

In contrast to the investigation into acclimation and storage temperatures by Law & Jerrett (1996 unpublished results) discussed in Chapter 3 there was no apparent detrimental effect of storing fillets under hyperbaric, hyperoxic conditions at temperatures well below half their acclimated temperature. There was an expectation that fillets stored at 2.0 °C would have had an increased rate of PM acidification due to cold-shock (Parry et al. 1987). Current theories suggest that at low temperatures membrane permeability is increased leading to an increase in energy expenditure on ion transport (Hochachka 1986; Hochachka et al. 1996). A study by Watabe et al. (1989) showed that Ca^{2+} uptake ability of the sarcoplasmic reticulum (SR) decreases at low storage temperature (0 °C) in the WM of plaice and red sea bream. The increased concentration of Ca^{2+} inside the myofibrils activated the myofibrillar Mg^{2+}-ATPase and the high rate of ATP consumption accelerated rigor mortis.

Differences in the storage protocol of the WM may explain these contrasting results. In the acclimation and storage temperature study by Law & Jerrett (1996) the WM was stored in RSW (refrigerated seawater) in the round, rather than in fillets in air in the current study. This would have resulted in a more rapid rate of cooling in RSW which may have augmented the effects of storage temperature. The slower rate of cooling in the current study may have allowed the WM metabolic processes to equilibrate more successfully to the lower temperatures reducing the effects of the cold-shock. It was possible that when the WM was stored at sub-optimum temperatures that

cold-shock did occur, i.e. the demand for ATP increased due to increased membrane permeability and decreased uptake of Ca^{2+} by the SR. However, the results suggested that ATP was able to be supplied via oxidative phosphorylation and was able to meet the increased demand without adding to the acidification of the WM. A study by Jerrett et al. (2002) investigating the effects of PM storage temperature on snapper (P. auratus) muscle supports this hypothesis. They found that while the WM pH was greater than ~7.2 (i.e. lactate levels were low) the WM was insensitive to the effects of low temperature (2 °C) storage. These results suggest that when the WM does not have to rely on anaerobic glycolysis for ATP generation it is in a "robust" state and can cope with stressors. Overall, the benefit to the WM when stored under hyperbaric, hyperoxic conditions far outweighed the detrimental effects of storage at low temperatures.

4.5.5 Post-mortem metabolism of rested ischemic white muscle stored under normobaric or hyperbaric conditions

The rate of pH decrease in the normobaric preparation was nearly three times as fast as WM held under hyperbaric conditions (Fig. 4.5). The large reduction in the rate of PM acidification in hyperbaric WM was attributed to the WM being able to respire aerobically (oxidative phosphorylation) due to oxygen being supplied to the mitochondria. A 12–27 h delay in depletion of ATP and glycogen and concomitant increases in lactate, P_i and creatine were also seen in the WM held under hyperbaric conditions supporting the hypothesis that the ATP demand was being met by a process other than anaerobic glycolysis.

Another study has investigated the effect of oxygen supply for modification of the PM metabolism in fish. Nagai et al. (2001) studied the effect of pre-harvest hydrostatic pressure treatment on PM rigor development in eels and tilapia. The air pressure was increased from 2 to 5 atm (absolute pressure) underwater with the live fish for 50 min (similar to the preliminary investigation described in the "Introduction"). This resulted in WM ATP levels being higher in pressure treated fish immediately after harvest, with [ATP] remaining high during PM storage at 3 °C. Times to reach full rigor mortis were delayed 23 h in eels and 6 h in tilapia. Different harvesting and handling procedures in the study made it difficult to make comparisons with the current work. It was thought that the continued supply of oxygen to the PM ischemic WM in

the current study proved more beneficial in delaying PM changes compared with increasing the oxygen content of the blood prior to harvesting as in the Nagai et al. (2001) study.

Natural exposure to high hydrostatic pressure

In nature some fish encounter high hydrostatic pressure (deep water) even though they would normally inhabit more shallow areas. An example of this is the migrating yellow freshwater eel (Anguilla anguilla). Fish that do not encounter such pressures naturally, such as trout (Oncorhynchus mykiss) are not able to physiologically tolerate these conditions experimentally. Exposure of live trout to 101 atm for 3 h induced a metabolic state resembling histotoxic hypoxia through a decrease in membrane fluidity (Sébert & Theron 2001). Yellow freshwater eels exposed to the same treatment show a less pronounced sensitivity to aerobic metabolism with their efficient anaerobic metabolism balancing the deleterious effects observed during the first hours of pressure exposure. Results suggest that oxidative phosphorylation is strongly altered in trout but not in eel. It has been hypothesised that the eel has supranormal mitochondria functioning at atmospheric pressure to counter this and cope with the high pressure environment encountered during its migration (Sébert & Theron 2001). In the current study the pressure used was only a fraction of that used in the high hydrostatic pressure investigations. From the results it would appear that oxidative phosphorylation was able to proceed in the PM WM under hyperbaric conditions. However, it was uncertain if the mitochondrial respiration was in some way damaged by the high level of oxygen in the tissue (i.e. became toxic similar to live trout under hydrostatic pressure) and therefore were not operating at their optimum. The fact that aerobic generation of ATP was only possible for ~27 h during PM storage suggests that this may have been the case. This possibility will be discussed further in a later section.

4.5.6 Oxygen penetration into the white muscle

An assumption made during the investigation was that oxygen was in fact being supplied to the ischemic WM held under hyperbaric, hyperoxic conditions. This assumption was made due to an observation during the tissue sampling process. To sample tissue, the chamber containing the ischemic hyperbaric tissue had to be depressurised. After ~27 h small bubbles began to appear in the tissue. These were

mainly in the circular muscle block encompassing the D-block. Either it took 27 h for the oxygen to saturate the tissue or, after 27 h the tissue could not use the oxygen (i.e. anaerobic glycolysis had taken over from oxidative phosphorylation) and was surplus to requirements. Several observations suggest that the latter of the two scenarios occurred. When an attempt was made to measure the level of oxygen in the WM with an oxygen electrode, very low levels were recorded until 27 h storage. Up until 12 to 27 h it was possible that the oxygen in the tissue was being consumed at a rate similar to that of supply, resulting in the low measured levels of oxygen. After 27 h there were high levels of oxygen measured in the WM, together with bubble formation, suggesting that the mitochondria were not able to consume the oxygen that was being supplied. After 27 h storage there were also significant decreases in WM [ATP] and [glycogen] and increases in [P_i] and [lactate]. A preliminary experiment showed that when WM was stored under hyperbaric conditions for 27 h, then depressurised and stored under normobaric conditions, there was no difference in WM pH profile compared with WM stored only under hyperbaric conditions (data not shown here). This also suggested that the WM only gained benefit from hyperbaric PM storage up to 27 h. The maintenance of the physiological state and ATP potential over the first 27 h storage supported the hypothesis that ATP was not being generated by anaerobic glycolysis. Instead the energy demand was more likely being fulfilled by oxidative phosphorylation due to the supply of oxygen to the WM.

4.5.7 Possible change in anaerobic metabolic rate of the hyperbaric white muscle

An interesting result was that once the level of the metabolites and biochemicals measured in the hyperbaric WM began to change during PM storage, the rates appeared very similar to that seen in the WM stored under normobaric conditions, with the possible exception of lactate (Fig. 4.6a). It has been reported that once the "switch-over" to anaerobic glycolysis has occurred the process progresses at a very high rate (Hochachka 1985). However, measurement of the hyperbaric WM pH showed that once anaerobic metabolism had commenced the rate of acidification was less than in normobaric WM. Therefore, it was possible that key regulation points of anaerobic glycolysis may have constrained the rate of lactate generation in some way in the WM held under hyperbaric conditions. This can possibly be explained by the Pasteur Effect.

When oxygen is introduced to anaerobic cells, the levels of all the glycolytic intermediates from fructose-1,6-bisphosphate onward decrease (see General Introduction, Fig. 1.2), while all of the earlier intermediates accumulate to higher levels. This finding is consistent with the idea that the metabolic flux through phosphofructokinase is specifically decreased in the presence of oxygen (Mathews & van Holde 1990). Another possibility is that the buffering capacity of the hyperbaric WM was greater than normobaric WM considering that the metabolite levels and their rate of change during PM storage were similar.

When the ATP levels in the WM were maintained at a steady level in the hyperbaric WM the actual turnover of ATP may have been very high or very low. It was possible that the aerobic ATP turnover rate may have had a large bearing on the consequent rate of anaerobic glycolysis. Because the rate of ATP utilisation sets the pace of all other chemical reactions (Bate-Smith & Bendall 1956), this hypothesis was supported.

If there is a deficit in ATP supply from the mitochondria, PCr catabolism can maintain the ATP concentration for a limited period and also provide a buffering role (consumes a H^+ for every ATP generated). This defence of ATP appears as an apparent "aerobic efficiency" but it is not clear what the actual rate of ATP turnover is. Aerobic generation of ATP is the far more efficient ATP generation process (compared with anaerobic metabolism), but in some situations such as hyperoxia, even the mitochondria can become less efficient, often to the detriment of the cell.

The potential problem with high levels of intracellular oxygen

Although oxygen is required by cells for aerobic respiration the concentration needed is very low. Oxygen pressures as low as 0.3-0.4 kPa (2-3 mmHg) have been observed in the intracellular microenvironment of mitochondria in tissues under normoxia (Gnaiger et al. 2000; Molé et al. 1999). High levels of oxygen in tissue can be hazardous to the life of the cell, due to oxygen's excellent electron-accepting properties with the formation of reactive oxygen species i.e. $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ (Skulachev 1996). It is these oxygen radicals that have been suggested to be responsible for reduced $\dot{M}O_2$ on aging (Camougrand & Rigoulet 2001). As mentioned earlier, bubbles (presumably oxygen) were observed in the WM of fillets held under pressure after ~27 h. This suggested that

after this time the WM was unable to respire aerobically even though there appeared to be sufficient oxygen available. Increase in [lactate], [P_i] and [creatine], along with a decrease in [ATP] did not occur until after 27 h, again suggesting a transition to anaerobic metabolism. It would appear that the mitochondria lose there ability to generate ATP together with a loss of supplemental ATP generating capacity from PCr. It is possible that the treatment was inefficient: delivery of oxygen to the WM may not have been fast enough to keep up with demand, or the removal of CO_2 was too slow, i.e. rate-limiting factors may have inhibited aerobic ATP generation. The mitochondria may have also become injured or may have been damaged by the high levels of oxygen in the tissue resulting in formation of damaging reactive oxygen species.

In the current study, when the WM was stored under hyperbaric, hyperoxic conditions there may have been a period when the concentration of oxygen diffusing into the WM was at the optimum for "healthy" aerobic generation of ATP via oxidative phosphorylation. As more oxygen diffused into the WM the concentration of oxygen may have become harmful to the mitochondria, with reactive oxygen species being produced. At this point the mitochondria themselves may have started "futile looping" (heat produced instead of ATP) in order to reduce the intracellular concentration of oxygen (Skulachev 1996). This strategy also leads to the consumption of ATP (this will be discussed in more depth in Chapter 6: Energy production in the white muscle).

While the ATP concentration was being maintained in the hyperbaric, hyperoxic WM it was possible that the ATP turnover rate was high and oxidative phosphorylation could not keep up with ATP demand. Initially any deficit in ATP production would have been corrected by utilisation of PCr stores. At the same time the intracellular oxygen concentration may have been such that it was damaging to the mitochondria and the mitochondria became net ATP consumers rather than producers (St-Pierre et al. 2000). Of course once this occurs the demand for ATP is so high that there is no alternative but to generate ATP anaerobically. The only way to accurately measure the true metabolic rate of the WM and determine whether the hyperbaric, hyperoxic WM has a low or high metabolic rate during the period of aerobic metabolism, is by measuring the heat output of the tissue, i.e. calorimetry. This area of research certainly warrants more attention in order to clarify what happens to the metabolic rate of the WM during PM storage.

Although it is of great interest to determine the rate of ATP consumption in the WM during PM storage it is also interesting to know the rate of ATP production by the mitochondria. In studies investigating the respiration rates of mitochondria, oxygen consumption is measured when saturating amounts of substrate (usually pyruvate in the case of teleost WM mitochondria; Moyes et al. 1989) are added in the presence of ADP (state III rate). After all the ADP has been phosphorylated the oxygen consumption rate is measured again (state IV rate). At physiological levels of ATP and ADP it has been observed that during rest and on recovery from exercise mitochondria respire at the state IV rate (Moyes et al. 1992). In the current study the WM was at rest pre- and post-harvest, with the muscle then excised from the whole animal. The process of filleting would have damaged some of the muscle, stimulated some contraction and therefore increased the demand for ATP. Despite this elevation from complete rest, it is suggested that when oxygen was available in the hyperbaric tissue the mitochondria would have still respired at the state IV rate (or slightly faster) rather than at the state III rate since the [ATP] in the WM was high (i.e. little ADP available). If this hypothesis is true then the rate of ATP turnover in the hyperbaric, hyperoxic WM during the aerobic phase would be relatively slow. It is possible that ATP levels were maintained for an extended period because the demand for ATP was being met solely by oxidative phosphorylation. Again, the only accurate way to measure this is by calorimetry.

4.5.8 Possible fuel alternatives for ATP production in the white muscle

Lipids are a major energy fuel for aerobic exercise in fish (Kieffer et al. 1998; van den Thillart & van Raaij 1995) and are stored in fish mainly as triglyceride (TG). Lipolysis of TG forms glycerol and free fatty acids (FFAs). FFAs cannot be oxidised (via β-oxidation) unless they are transported into the mitochondria, for which carnitine is the vehicle through the formation of acyl-carnitine. β-oxidation produces acetyl-CoA to fuel the Krebs cycle (Wang et al. 1994a).

The amount of lipid stored in teleost WM is relatively low compared with storage in the red muscle due to the red muscle being recruited for aerobic sustainable swimming (van den Thillart & van Raaij 1995). The mullet used in the current study were fed on an alginate-bound diet as well as a commercial pelletised diet high in oil content formulated for high growth rates. The mullet also had large visceral fat deposits

suggesting that the diet had increased the "normal" lipid levels in the mullet. It has been reported that mitochondria from red muscle in carp can use pyruvate and fatty acids (fatty acyl carnitines) equally well as aerobic fuels whereas in the WM the mitochondria oxidise fatty acyl carnitines at 35-70% the rate of pyruvate, depending on chain length (Moyes et al. 1989). Therefore it is possible for FFAs to be utilised in the WM albeit at a slower rate than pyruvate. Moyes et al. (1992) studying the role of mitochondria in trout WM found that free fatty acids (FFAs) inhibit pyruvate dehydrogenase. If FFAs were able to be utilised in the hyperbaric WM instead of pyruvate for aerobic energy supply, less CO_2 would be produced due to a decrease in the oxidative decarboxylation of pyruvate to acetyl-CoA (one molecule of CO_2). Thus, CO_2's effect as a weak acid would be less and would result in a decrease in the rate of pH rundown in the WM.

In the study by Wang et al. (1994) the effects of exercise in trout muscle were investigated and they found that there was no change to the redox state of the WM during or after exercise suggesting that the cytoplasmic compartment of WM remained well oxygenated while there was production of lactate. This challenged the view of "anaerobic" production of lactate, i.e. limitation of O_2 supply is not a determinant of lactate production during intense exercise. In the same study there were increases in acyl-carnitines during and after exhaustive exercise indicative of utilisation of FFAs as an oxidative fuel for ATP generation. It has also been reported in mammals that an increase in the availability of FFAs actually depresses carbohydrate utilisation (i.e. glycolysis, Rennie et al. 1976). In the current study it is possible that while the WM was able to respire aerobically under hyperbaric conditions (1-27 h), fatty acids were able to be utilised to produce ATP, in turn, sparing carbohydrate sources. However, further analysis of lipid levels etc, would need to be carried out to confirm this.

4.5.9 High pressure technology

Although there are many studies demonstrating the effects of pressure on PM muscle the focus of the investigations is usually the manipulation of key enzymes and proteins to improve product quality and shelf-life (Ashie & Lanier 2000; Horgan & Kuypers 1983; Ko et al. 1991). It could be argued that was the primary objective of the current work. However, the focus of the present study came from a different approach: pressure was to be used in order to deliver a limiting substrate to the ischemic WM. It was

uncertain whether the pressure used in the standard storage protocol would have been high enough to have any effect at the enzyme level. Conventional high pressure treatments use pressures many hundreds of times greater than those used in the hyperbaric, hyperoxic treatment in the current study. In addition, pressures used in these experiments were similar to those experienced by these species in nature. From the results it would appear that the over-riding effect of the hyperbaric treatment was allowing the WM to generate ATP aerobically for ~27 h during PM storage.

4.5.10 Clinical applications of hyperbaric treatment

Hyperbaric oxygen (HBO) therapy, in combination with cold storage, has been reported to also improve the survival rate of experimental epigastric and groin skin flaps of rats (Angel et al. 1994; Tai et al. 1992). It was suggested that protection of the antioxidative enzymes by hypothermia and inhibition of xanthine oxidase activity by HBO improved skin flap survival. In the current application it was hypothesised that the reduction in the rate of acidification in the ischemic WM preparation and the delay in onset of anaerobic glycolysis was, in part, due to the increased amount of oxygen in the tissue (allowing aerobic glycolysis to continue for a longer period) and a reduction in activity of key glycolytic enzymes because of this.

4.5.11 Questions to arise from these experiments

As mentioned earlier, glycogen was still present in the WM at significant levels toward the end of the storage period. However, if this substrate was not available to the cells for energy metabolism this may have been another limiting factor to why the WM lost viability even though we assume that it was supplied with the appropriate substrates (i.e. oxygen and glycogen).

Even though the rate of acidification in the hyperbaric WM preparation was reduced by aerobic generation of ATP supplementing energy requirements, a reason for the loss of viability, even in the presence of (assumed) sufficient oxygen is unclear. The major question to arise from these experiments was why does the PM WM stored under hyperbaric conditions still have to change from generating ATP aerobically to generating ATP anaerobically? Why can the WM not continue to efficiently generate ATP when there is substrate available (glycogen) and oxygen being supplied to the WM? The rate at which oxidative phosphorylation proceeds may only be partially

effective in maintaining ATP levels. Above a WM pH of 7.0 the deficit might be made up by PCr utilisation, below pH 7.0 generation of ATP is limited to anaerobic glycolysis. Therefore, is WM pH a limiting factor as to why aerobic generation of ATP ceases?

Once PCr is depleted can glycogen be completely utilised as a glucose substrate for aerobic metabolism? From the results it appears that there are substantial levels of glycogen in the WM that do not become entirely depleted, even at the end of the storage period. However, if this substrate is not available to the cells for energy metabolism this might be another limiting factor to why the WM loses viability even though we assume that it is supplied with the appropriate substrates (i.e. oxygen and glycogen).

Is the rate of aerobic ATP generation diffusion limited? As discussed previously there may be an optimum concentration of oxygen in the tissue that can sustain aerobic metabolism but as storage continues and more oxygen diffuses into the tissue this may change. As the mitochondria are the site of aerobic generation of ATP it may be that they hold the key as to the reason for the inevitable progression of PM changes in the WM. As discussed above, the high levels of oxygen and/or CO_2 in the WM stored under hyperbaric, hyperoxic conditions may in fact poison the mitochondria giving the WM only one option for ATP generation: anaerobic glycolysis. The questions raised during these experiments regarding the role of mitochondria in the PM metabolism of hyperbaric WM and why they are not able to maintain sufficient levels of ATP in the WM during storage will be addressed further in Chapter 6.

Although determining why the hyperbaric WM still progressed through the typical PM changes was a major focus of the thesis it was also important to transfer the knowledge gained from the PM studies in the yellow-eye mullet to other more commercially valuable New Zealand teleost species. In this regard the following chapter extends the investigation of PM storage under hyperbaric conditions to chinook salmon (Oncorhynchus tshawytscha) and snapper (Pagrus auratus).

4.6 SUMMARY

Storage of rested ischemic WM under hyperbaric conditions (620 ± 10 kPa pressure with oxygen flow at 50 mL/min ± 1.25 %) at the optimum storage temperature significantly modified the PM metabolism of the WM. The fall in WM cut-surface pH was 2.7 times slower than in normobaric WM, and there was a 11-27 h delay in changes to key WM metabolites. The delay in lactate, P_i and creatine accumulation and ATP and glycogen depletion suggested that energy supply for the tissue during this delay period was supplied through aerobic means rather than a substantial slowing of anaerobic metabolism. Once changes to the key metabolites (ATP, lactate etc) in the WM began to occur, the apparent rates of accumulation/depletion were similar to those seen in the WM held at normobaric pressure apart from lactate accumulation which was slower. Again this suggests that the rate of anaerobic metabolism had not been greatly slowed by the hyperbaric treatment, instead the treatment allowed the WM to respire aerobically for a period. From these findings and the observation that bubbles formed in the WM after 27 h storage, it was assumed that the WM was indeed being supplied with oxygen. It remains unclear why the WM made the switch to anaerobic metabolism when ATP levels were being defended and there was still adequate substrate (glycogen) available and oxygen was presumably in excess. Although aerobic metabolism was supplying the WM with ATP, the WM pH still dropped, possibly due to increased CO_2 concentration. This drop in pH could have favoured activation of enzymes in anaerobic pathways and inhibited those in aerobic routes. Overall, hyperbaric storage of WM allowed the rested muscle to respire aerobically for a 11-27 h period, prior to switching to anaerobic supply of energy.

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CHAPTER 5

The effect of supplementary, hyperbaric oxygen supply on the post-mortem metabolism of rested, ischemic white muscle from chinook salmon (Oncorhynchus tshawytscha) and snapper (Pagrus auratus)

5.1 ABSTRACT

The objective of the study was to extend the PM investigation of PM mullet metabolism (Chapter 4) to chinook salmon (Oncorhynchus tshawytscha) and snapper (Pagrus auratus). These commercially important species have differences in WM structure and function that were likely to be reflected in the response of the WM to hyperbaric treatment. Ischemic WM was stored under hyperbaric conditions (620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%) and compared with tissue stored under normobaric conditions (humidified oxygen flow set to 50 mL/min ± 1.25%) at the optimum storage temperature for the species. WM pH was measured during PM storage along with key metabolites (lactate, P_i, ATP, and creatine). The rate of acidification in the WM stored under hyperbaric conditions was reduced by two-thirds in comparison with WM stored under normobaric conditions. Snapper WM gained the most benefit from PM hyperbaric storage with ATP depletion and onset of anaerobic glycolysis being delayed for ~37 h. In mullet the delay was ~27 h (Chapter 4) and in salmon only ~12 h. The poor physical condition of the salmon significantly affected the PM metabolism of the WM. The associated low glycogen and lipid levels in the WM may have limited the substrate required for both anaerobic and aerobic metabolism. In addition mobilisation of WM protein due to malnourishment may have lead to reduced buffering capacity of the salmon WM.

The large benefit snapper WM gained from hyperbaric storage and the rate of PM metabolism in all three species may be directly linked to the rate of WM recovery from burst exercise. Slow recovery may translate to slow and prolonged ATP generation and slowed rate of acidification in PM rested WM.

5.2 INTRODUCTION

As hyperbaric, hyperoxic storage of yellow-eye mullet WM was successful in retarding PM metabolic changes in the WM it was of interest to extend the investigation to two commercially important New Zealand species: chinook salmon (Oncorhynchus tshawytscha) and snapper (Pagrus auratus).

Salmon and snapper prefer very different habitats. Chinook salmon are a temperate, anadromous species native to the cool waters of the Northern Hemisphere (Paul 2000). New Zealand snapper are a sub-tropical/temperate marine species found along the coasts of the North Island of New Zealand and extend south to Tasman Bay. Chinook salmon are exposed to temperatures ranging from 0 to 24 °C (Brett 1956) with an optimum temperature of 10 to 15 °C (Burrows 1972). Snapper are typically found at temperatures ranging from ~12 to 25 °C (Jerrett et al. unpublished data). From previous investigations in the author's laboratory it was known that the optimum storage temperature for the WM is different for chinook salmon, snapper and yellow-eye mullet (see Chapter 3). This was seen as a reflection of environmental preferences of each species. This difference in water temperature preference was reflected in their optimum storage temperature (2-4 °C for salmon, Jerrett et al. 2000; ~7-10 °C for snapper, Jerrett et al. 2002 in press; ~8 °C for mullet, Jerrett et al. unpublished data).

The functional differences between salmon and snapper are also quite marked. Salmon are from the family Salmonidae and are, in many respects, a "high performance" fish (Milligan 1996). They are able to maintain substantial swimming speeds against currents (i.e. swimming up river), but also retain the ability for burst activity. Snapper are from the family Sparidae and are a demersal reef species that swim primarily at a cruising speed (not at speeds as great as salmon). Salmon muscle has been described as a "mosaic" having mixed white and red fibres with both fibre types recruited for swimming at sub-burst velocities (Moyle & Cech 1988). However, Johnston et al. (1975) and Johnston & Moon (1980) showed that the myofibrillar ATPase activity of the smaller fibres (supposed red fibres) of rainbow trout and brook trout were essentially similar to that of the larger fibres (white fibres) and very different from that of red muscle, though the smaller fibres had greater succinic dehydrogenase activity than the large. These smaller fibres were therefore characterised as growth stages, present in greater numbers in fish of smaller size classes, rather than distinct red

fibres (Kiessling et al. 1991). In snapper, the red and white muscles are separate with the WM being poorly vascularised. Burst exercise in this species must be fuelled primarily by anaerobic glycolysis in the WM. When salmon swim aerobically the majority of their energy production is through lipid metabolism in the red muscle, while burst activity relies heavily on the breakdown of glycogen via anaerobic glycolysis (van den Thillart & van Raaij 1995). Correspondingly, there are high levels of lipid in the red muscle (40 μmol/g, stored primarily as triglycerides) and high glycogen reserves in the WM (40 μmol/g; Moyes & West 1995). Unlike salmon, slow-speed manoeuvring and aerobic swimming in snapper is achieved by their strong pectoral fins, not by use of the whole body. Even though snapper have lateral red muscle which is recruited for aerobic cruising, they are much leaner (less storage of lipid in the muscle) than salmon and do not possess a mosaic muscle structure.

It is generally well accepted that during recovery from burst exercise salmonid muscle glycogen synthesis occurs via lactate-based in situ glycogenesis (Milligan & Girard 1993; Milligan 1996; Wang et al. 1994). In a study by Wang et al. (1994) 10-20% of the lactate produced was released into the blood where it could be used in the liver and heart. However, some of this lactate could be taken back up into the muscle for glycogenesis. The somewhat more "aerobic" nature of salmon WM suggests that the muscle is better perfused and, therefore, end products of metabolism, such as H^+, CO_2, could be removed from the tissue via the circulation and the pH of the WM may remain stable. This means that while salmon are swimming at relatively high speed, WM can still be recruited for regular short burst activity even though there may have recently been high levels of lactate in the muscle. In contrast, snapper have poorly vascularised WM (personal observation) and therefore rapid recovery from burst exercise by releasing waste products into the blood stream is unlikely. Overall, bursts would be of short duration followed by a relatively long recovery period. When it comes to the ischemic preparation where there is no waste removal, H^+ would be able to build up in the muscle, disrupting cellular homeostasis.

The main objective of this study was to determine the effect that hyperbaric storage conditions had on the PM metabolism of rested ischemic WM preparations from chinook salmon and snapper. This was compared with the PM metabolism of rested ischemic WM that was held under normobaric conditions. The methods used in the current study were the same as Chapter 4 for the similar investigation in yellow-eye

mullet. Due to the difference in WM structure, and proposed strategies for coping with metabolic end-products in salmon and snapper, it was hypothesised that the effect of holding WM under hyperbaric conditions might be more beneficial in delaying the onset of accumulation of metabolic end-products in salmon because of their "high performance" ability and aerobic capacity.

5.3 MATERIALS AND METHODS

5.3.1 Experimental animals

Juvenile female snapper (Pagrus auratus) were sampled in December 1998 with the mean seawater temperature for the 14 d period prior to sampling being was 17.7 ± 0.2 °C (± SEM).

Female chinook salmon, (Oncorhynchus tshawytscha) were sampled in January 1999 with the mean seawater temperature for the 14 d period prior to sampling being 16.9 ± 0.2 °C (± SEM).

5.3.2 Rested harvesting

Both salmon and snapper were sampled using rested harvesting techniques. Salmon were anaesthetised at a concentration of 19.0 ± 0.5 mg/L (ambient water temperature 17.3 ± 0.1 °C) and snapper at 17.0 ± 0.5 mg/L (ambient water temperature 17.4 ± 0.1°C). The appropriate concentrations for anaesthetisation with AQUI-S^{TM} Plus were determined for each species in previous investigations. After 35 min the salmon were anaesthetised (insensitive to contact of the gill lamellae), however, snapper took 65 min before they were anaesthetised. Once in the anaesthetised state, experimentation could begin. Experimental timing was recorded from the introduction of the anaesthetic and the timing of individual measurements were recorded to the nearest minute.

Once the fish were anaesthetised a mixed venous blood sample was taken and the pH was measured along with the lactate and glucose (see Chapter 2: "Blood sampling and pH measurement). The fish were pithed immediately after the blood sample was taken.

5.3.3 Fillet storage

Fillet storage was the same as the standard storage protocol described in Chapter 4 for yellow-eye mullet: left-hand side fillets were stored under hyperbaric conditions (620 ± 10 kPa with oxygen flow set to 50 mL/min ± 1.25%) compared with the right-hand side fillets stored under normobaric conditions (oxygen flow set to 50 mL/min ± 1.25%). Salmon ischemic WM was stored at 4.0 ± 0.1°C, and snapper at 8.5 ± 0.1°C. These

storage temperatures were shown to be the optimum storage temperatures for the species concerned at summer acclimated temperatures (Jerrett et al. 2000, 2002; see Introduction).

5.3.4 White muscle pH measurement and metabolite determinations

The cut-surface WM pH measurements and WM samples were taken from salmon and snapper fillets prior to going into storage and then 13, 27, 37, 50, 55 and 74 h after storage began using the same method described in Chapter 2. Lactate, ATP, P_i, and creatine concentrations were measured in the WM samples with methodology as described in Chapter 2. Glycogen was not measured in the WM of snapper or salmon as it was decided after carrying out the mullet extracts that the glycogen assay was too variable, time consuming and expensive.

5.3.5 Statistical analysis

All times, pH values and biochemical values stated in the text are the mean ± standard error of the mean (SEM). Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows Version 6.00 (Copyright 1986-2000 SPSS Inc.) and Microsoft® Excel 2000.

5.4 RESULTS

It was important for fish to be in the same "state" of anaesthesia prior to handling. Snapper had to be left in the anaesthetic for a longer period than the salmon taking almost twice as long to become anaesthetised. The snapper were therefore pithed later (Table 5.1).

5.4.1 Post-harvest condition

Salmon and snapper were of similar size, however, the snapper were in significantly better condition than salmon (Table 5.1). In particular, snapper had a higher HSI than salmon, the HSI for salmon being poor (<1; Love 1980). The condition factor (CF) of the salmon was not poor (<1), however, was also lower than the snapper.

There was no significant difference in the blood pH measurements between salmon and snapper. In both salmon and snapper the blood lactate concentrations were low with blood glucose levels being significantly higher in the snapper (Table 5.1).

Table 5.1. *Size, condition and whole blood measurements from salmon and snapper.*

	Salmon	Snapper
Time to pithing (h)	0.62 ± 0.04	1.11 ± 0.08*
Weight (g)	538.7 ± 28.9	634.3 ± 52.6
Length (mm)	346 ± 5	304 ± 9
CF[1]	1.30 ± 0.03	2.24 ± 0.08*
HSI[2]	0.77 ± 0.04	1.81 ± 0.19*
Blood pH	7.44 ± 0.07	7.59 ± 0.08 (n=4)
Blood lactate (mmol/L)	2.0 ± 0.4	2.0 ± 0.2 (n=4)
Blood glucose (mmol/L)	3.7 ± 0.3	6.4 ± 0.9 (n=4)*

[1]Condition factor (CF) = weight (g)/length (mm3) x 100000 (Love 1980).
[2]Hepatosomatic index (HSI) = liver weight (g)/weight (g) x 100 was (Love 1980).
Values are the mean ± SEM, n = 5 unless otherwise stated
* Significantly different to salmon value ($P < 0.05$; two-tail Student's t-test assuming unequal variance).

The initial WM pH for salmon was 0.24 pH units lower than snapper (Table 5.2) and also had significantly higher levels of P_i. The other metabolites measured in the WM prior to storage were of similar concentration between the two species.

Table 5.2. *Pre-storage white muscle metabolite levels in salmon and snapper.*

	Salmon	Snapper
Sampling time (h)	1.18 ± 0.14	1.76 ± 0.14*
White muscle pH	7.48 ± 0.02 (n=5)	7.72 ± 0.02* (n=5)
Lactate μmol/g	20.6 ± 2.3	16.5 ± 2.4
ATP μmol/g	7.1 ± 0.4	9.3 ± 1.4
P_i μmol/g	34.5 ± 4.8	20.9 ± 3.6*
Creatine μmol/g	16.5 ± 1.0	16.7 ± 2.5

Values are the mean ± SEM, n = 4 unless otherwise stated.
* Significantly different to salmon value ($P < 0.05$; one-tail Student's t-test assuming unequal variance).

5.4.2 Post-mortem white muscle pH profiles

In salmon WM stored under normobaric conditions the cut-surface pH dropped rapidly over the first 13 h of and then reached the ultimate pH (~6.65) between 27 and 37 h (Fig. 5.1a). In contrast, the snapper WM pH declined at a steady rate reaching the ultimate pH of ~6.55 after 37 h storage (Fig. 5.1b).

The WM cut-surface pH profiles measured in salmon and snapper WM during hyperbaric storage are also shown in Figs. 5.1a and 5.1b, respectively. In salmon there was a rapid decrease in pH over the first 13 h of storage with a further decrease after 37 h down to the ultimate pH. In snapper the fall in WM pH was steady over the experimental period, however, after 74 h the hyperbaric snapper WM pH was still ~0.4 pH units higher than the ultimate pH measured in the WM held under normobaric conditions. If the rate of WM acidification remained constant in the hyperbaric WM the ultimate pH value would have been reached after ~106 h. Continued storage of snapper preparations was, unfortunately, not possible due to a lack of tissue.

During the sampling process it was also noted that bubbles in the WM stored under hyperbaric conditions started to appear after 27 h in both salmon and snapper.

5.4.3 Post-mortem white muscle metabolite profiles

Lactate

Under normobaric PM storage conditions the lactate concentration in salmon WM increased rapidly from the resting pre-storage level peaking at ~60 μmol/g muscle mass after 13 h of storage. Lactate accumulation in the appeared slower than in salmon (Fig 5.2b). After a rapid rise in lactate concentration from resting levels up to ~70 μmol/g over the first 37 h storage, there was a further increase up to ~80 μmol/g muscle mss between 50 and 74 h.

Salmon WM stored under hyperbaric conditions showed a steady increase in WM [lactate] over the first 50 h of storage (Fig. 5.2a). The lactate level then reached a plateau at ~60 μmol/g muscle mass (similar to the normobaric plateau) for the remaining storage period. In contrast, the snapper hyperbaric WM showed a delay in lactate accumulation for between 13 and 37 h (Fig. 5.2b). As observed in the WM pH measurements of snapper, WM [lactate] in the hyperbaric preparation did not reach the same plateau concentrations seen in salmon over the 74 h storage period. However, it was assumed that the lactate levels in hyperbaric snapper WM would have reached ~75 μmol/g muscle mass if storage had been continued and the tissue had behaved in a similar way as the salmon.

The relationship between WM pH and WM [lactate] in both snapper and salmon is shown in Figs. 5.3a & b. In both species (excluding salmon normobaric WM) the relationship observed was a significant negative correlation ($P < 0.001$). Measurement of the WM cut-surface pH was, therefore, a good predictor of WM [lactate]. By performing an analysis of covariance (Zar 1984) the regression slopes between salmon and snapper were found to be not significantly different from each other. In the normobaric salmon WM there was no clear relationship between WM pH and lactate concentration (Fig. 5.3a). At a WM pH of ~6.65 (ultimate pH) the WM lactate concentration ranged from 30 to 75 μmol/g muscle mass in normobaric salmon WM. In this case the WM pH was not an accurate predictor of WM [lactate].

ATP

ATP levels in salmon WM held under normobaric conditions decreased rapidly from the start of PM storage and reached levels of <1 μmol/g muscle mass after 50 h (Fig. 5.4a). In the normobaric snapper WM the [ATP] decreased in a similar way to WM pH in that there was a steady decline over the first 37 h of storage down to ~1 μmol/g muscle mass for the remaining storage period (Fig. 5.4b).

Hyperbaric salmon WM showed no delay in ATP depletion during PM storage (Fig. 5.4a). ATP levels decreased rapidly down to <1 μmol/g muscle mass over the first 50 h. In snapper WM held under hyperbaric conditions there was at least a 12 h delay in ATP depletion (Fig. 5.4b; c.f. lactate accumulation, Fig 5.2b). ATP levels then slowly decreased over the storage period. Even though the ATP measurements were quite variable, the mean ATP concentration did not drop below 5 μmol/g muscle mass until after ~57 h storage . By the end of the storage period [ATP] had not dropped to <1 μmol/g muscle mass, but it was assumed that this would have occurred if storage had continued.

The relationship between WM pH and ATP concentration in both salmon and snapper held under hyperbaric and normobaric conditions is shown in Figs 5.5a and 5.5b, respectively. In salmon there was little difference in the relationship between the two storage protocols (Fig 5.5a). WM pH was a relatively good predictor of [ATP] in salmon WM as shown by the r^2 values of the regression equations (see legend of Fig. 5.5). In snapper WM there was a considerable difference between the pH and ATP relationships of the two storage protocols (Fig. 5.5b). Snapper WM held under hyperbaric conditions had higher pH values for the equivalent ATP concentration compared with WM held under normobaric conditions. The strength of the relationship under hyperbaric conditions was poor (r^2 = 0.22. see legend Fig. 5.5), whereas normobaric storage resulted in a strong relationship (r^2 = 0.85).

Inorganic phosphate

Salmon WM stored under normobaric conditions showed a rapid increase in [P_i] over the first 13 h of storage (Fig 5.6a). The [P_i] then reached a plateau between 13 and 37 h and then increased to a peak of ~60 μmol/g muscle mass after 74 h storage. In snapper WM stored under normobaric conditions the P_i accumulation showed a similar pattern

as the increase in [lactate]: there was a steady increase over the first 37 h and then an increase to ~60 μmol/g muscle mass after 74 h storage (Fig 5.6b).

In salmon WM held under hyperbaric conditions there was a ~13 h delay in accumulation of [P_i] (Fig. 5.6a). Over the remaining storage period P_i levels rose steadily to ~55-60 μmol/g muscle mass. Again, there was substantial variation in [P_i] and therefore only the major trend of an increase in [P_i] over the storage period for both normobaric and hyperbaric preparations will be discussed. In the hyperbaric snapper WM there was a ~37 h delay before P_i began to accumulate (Fig 5.6b). [P_i] then rose to a peak of ~40-45 μmol/g muscle mass after 50 h. [P_i] would have been expected to rise to similar levels as in normobaric WM if storage had continued.

The relationship between WM pH and P_i concentration in both salmon and snapper held under hyperbaric conditions and at normobaric pressure are shown in Figs 5.7a & b. The relationship was very poor in both storage protocols for salmon. In the normobaric WM once the ultimate pH had been reached (~6.6) there was a large amount of variation in the [P_i]. In hyperbaric WM high levels of P_i were often associated with WM pH values above 7.0 (Fig. 5.7a). The relationship between WM pH and [P_i] in normobaric snapper WM was more similar to that shown in mullet WM (Fig. 5.7b), with hyperbaric snapper WM showing a weaker relationship.

The relationship between WM [P_i] and WM [ATP] in salmon and snapper is (Fig. 5.8a & b, respectively). In salmon WM stored under normobaric conditions the majority of [P_i] concentrations above 40 μmol/g muscle mass corresponded to very low levels of ATP. In hyperbaric WM the relationship was better resolved with intermediate levels of ATP corresponding with intermediate levels of P_i, but once ATP had been depleted the [P_i] continued to rise. In snapper, once again, there was a distinct difference in the [P_i]/[ATP] relationship between normobaric and hyperbaric WM (Fig. 5.8b). In normobaric WM the relationship was more similar to that in hyperbaric salmon WM. In hyperbaric snapper WM high levels of P_i corresponded to relatively high levels of ATP.

Creatine

Salmon WM stored under normobaric conditions showed an immediate accumulation of creatine over the first 12 h and rose to ~23 μmol/g muscle mass (Fig 5.9a). The pre-

storage [creatine] for normobaric snapper WM was 16.7 ± 2.5 μmol/g muscle mass (see Table 5.1). This may have been over-estimated, suggested by the large drop in [creatine] to ~10 μmol/g in the hyperbaric WM after 12 h. A more likely pre-storage concentration would have been similar to the 12 h value of 10.3 ± 1.8 μmol/g muscle mass. This value has been plotted on the graph instead of the measured concentration (Fig. 5.9b). With this revised value the [creatine] in normobaric WM rose rapidly over the first 12 h of storage to ~20-22 μmol/g muscle mass. WM from both salmon and snapper stored under hyperbaric conditions showed a 12 h delay before creatine levels rose (Fig. 5.9a & b). Due to the methodology problem with the creatine assay (refer: Chapter 2), once the creatine concentration had reached 20 μmol/g muscle mass during PM storage further data points were omitted from the graphs.

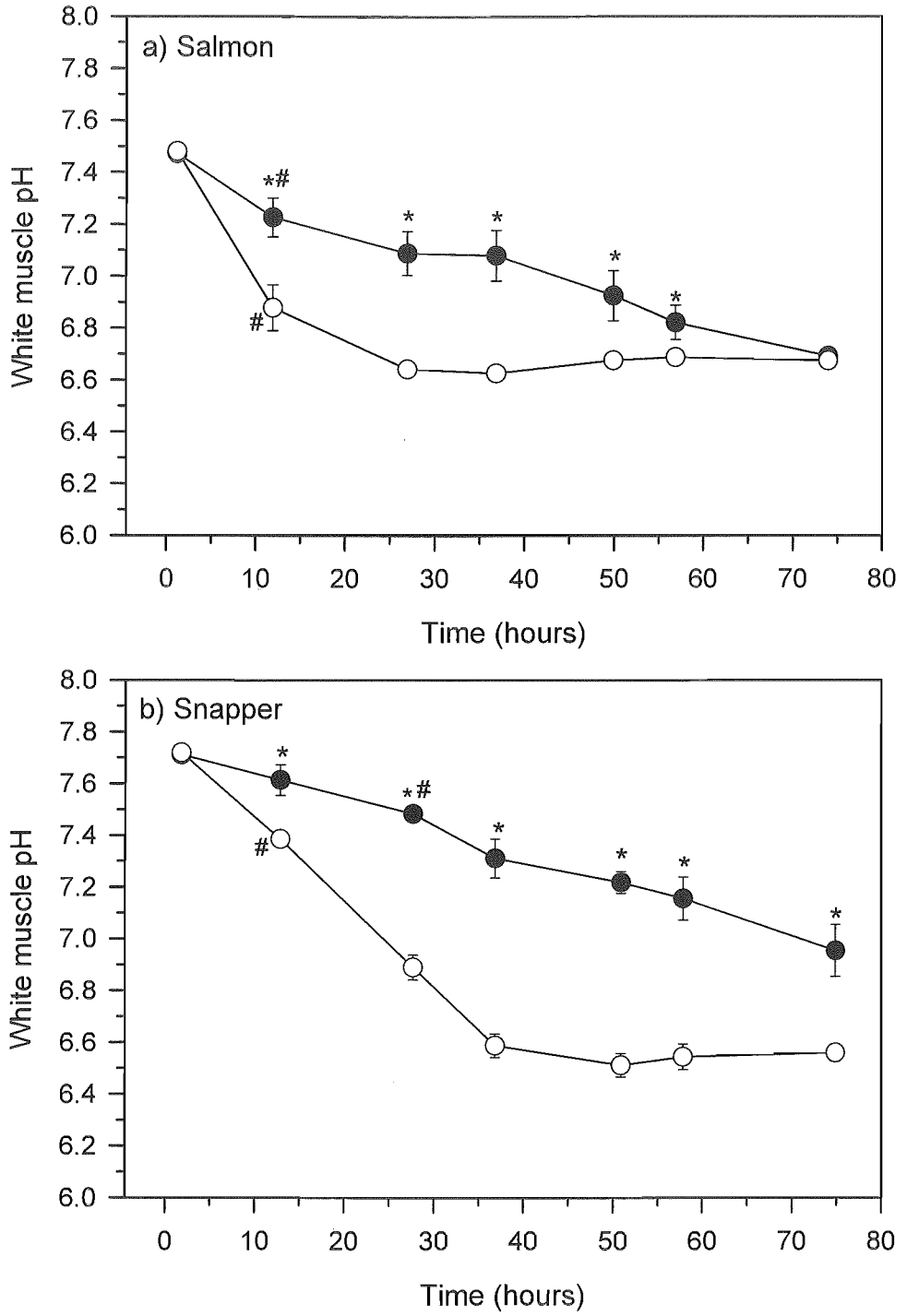

Figure 5.1: *Post-mortem cut-surface pH of the epaxial white muscle from a) rested chinook salmon, and b) rested snapper stored under normobaric conditions with oxygen flow 50 mL/min ± 1.25 % (○) or under hyperbaric conditions 620 ± 10 kPa oxygen flow 50 mL/min ± 1.25 % (●) in a water bath at 4.0 ± 0.1 °C for salmon and 8.5 ± 0.1 °C for snapper. Values are the mean ± SEM; n = 5. * Significantly different from the corresponding normobaric value at the same sampling time (Sign test; P < 0.05). # Significantly different from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

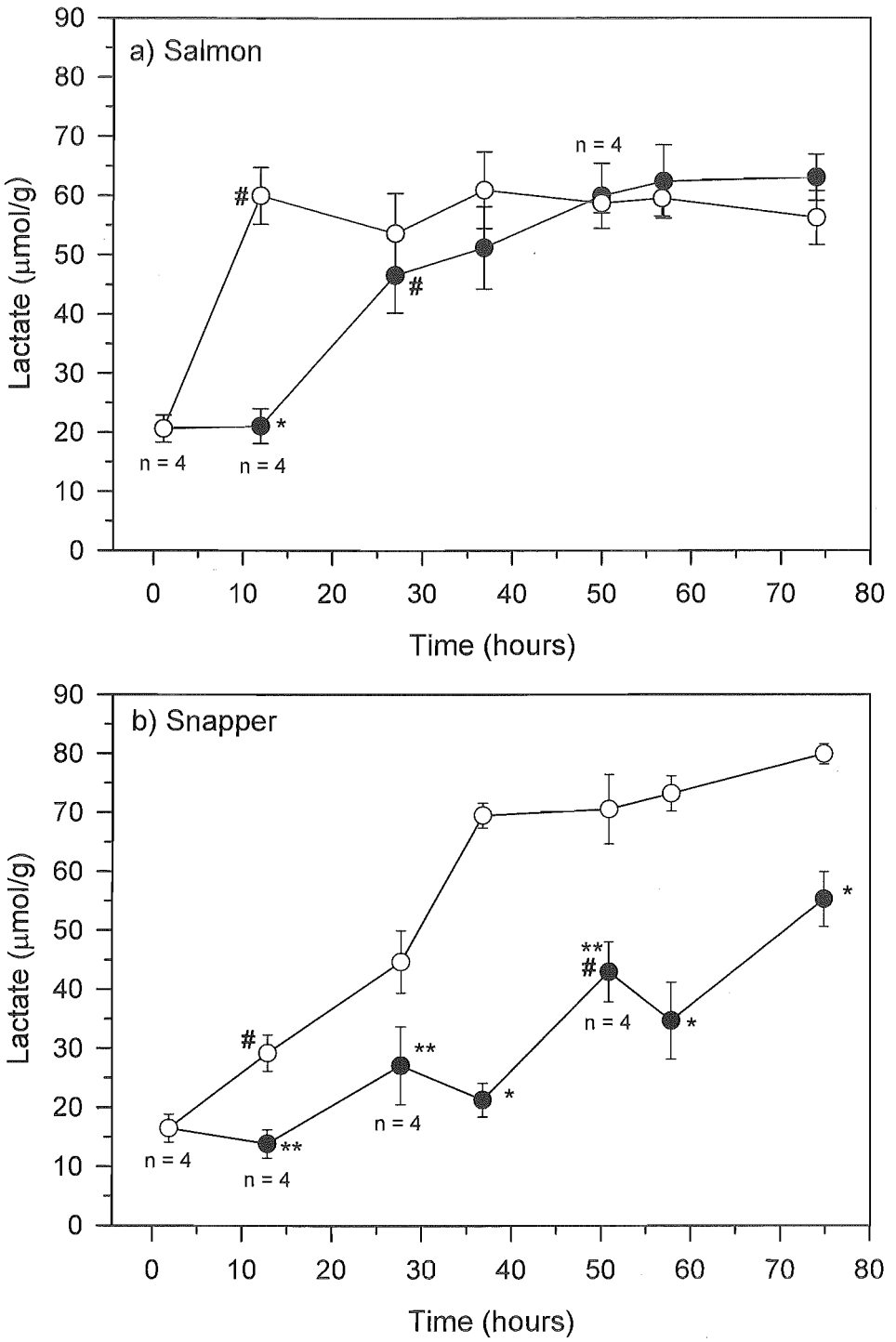

Figure 5.2: *Post-mortem lactate content of the epaxial white muscle of a) chinook salmon and b) snapper stored under hyperbaric (●), or normobaric (○) conditions. For storage details see Fig. 5.1 legend. Values are the mean ± SEM, n = 5 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. ** Significantly different from the corresponding normobaric value at the same sampling time (Sign test P = 0.06 as n = 4). # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

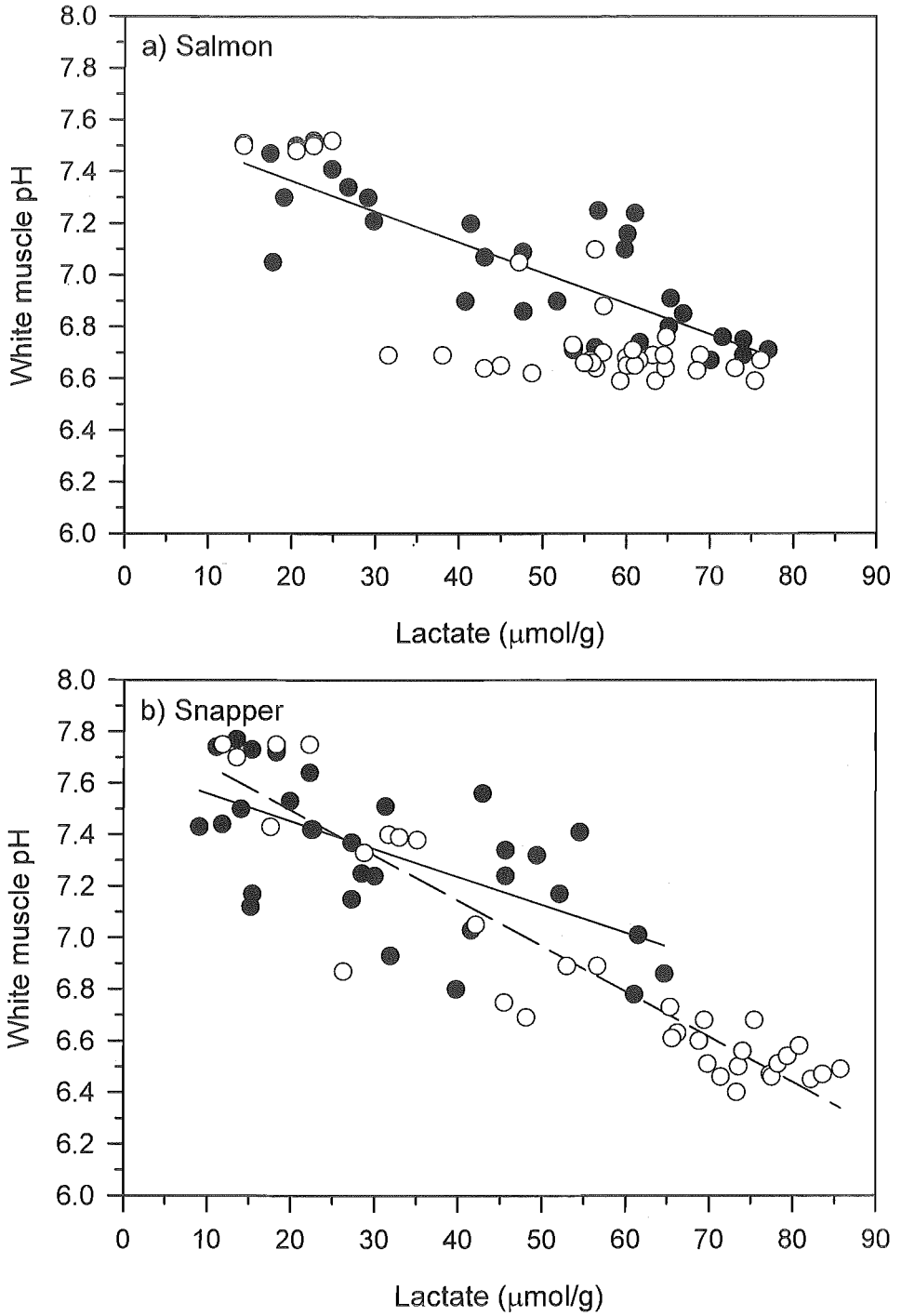

Figure 5.3: *Relationship between post-mortem cut-surface pH and [lactate] from the epaxial white muscle of a) salmon, and b) snapper stored under hyperbaric (●) or normobaric (○) conditions. The pH/[lactate] relationship of hyperbaric salmon white muscle (solid line) is described by the linear regression equation* $y = -0.012x + 7.60$, $r^2 = 0.0.67$. *The pH/[lactate] relationship in normobaric salmon white muscle was poor and therefore no regression line was fitted to the data. The pH/[lactate] relationship of hyperbaric snapper white muscle (solid line) is described by the linear regression equation* $y = -0.011x + 7.67$, $r^2 = 0.41$; *for normobaric white muscle (○, dashed line)* $y = -0.018x + 7.85$, $r^2 = 0.87$.

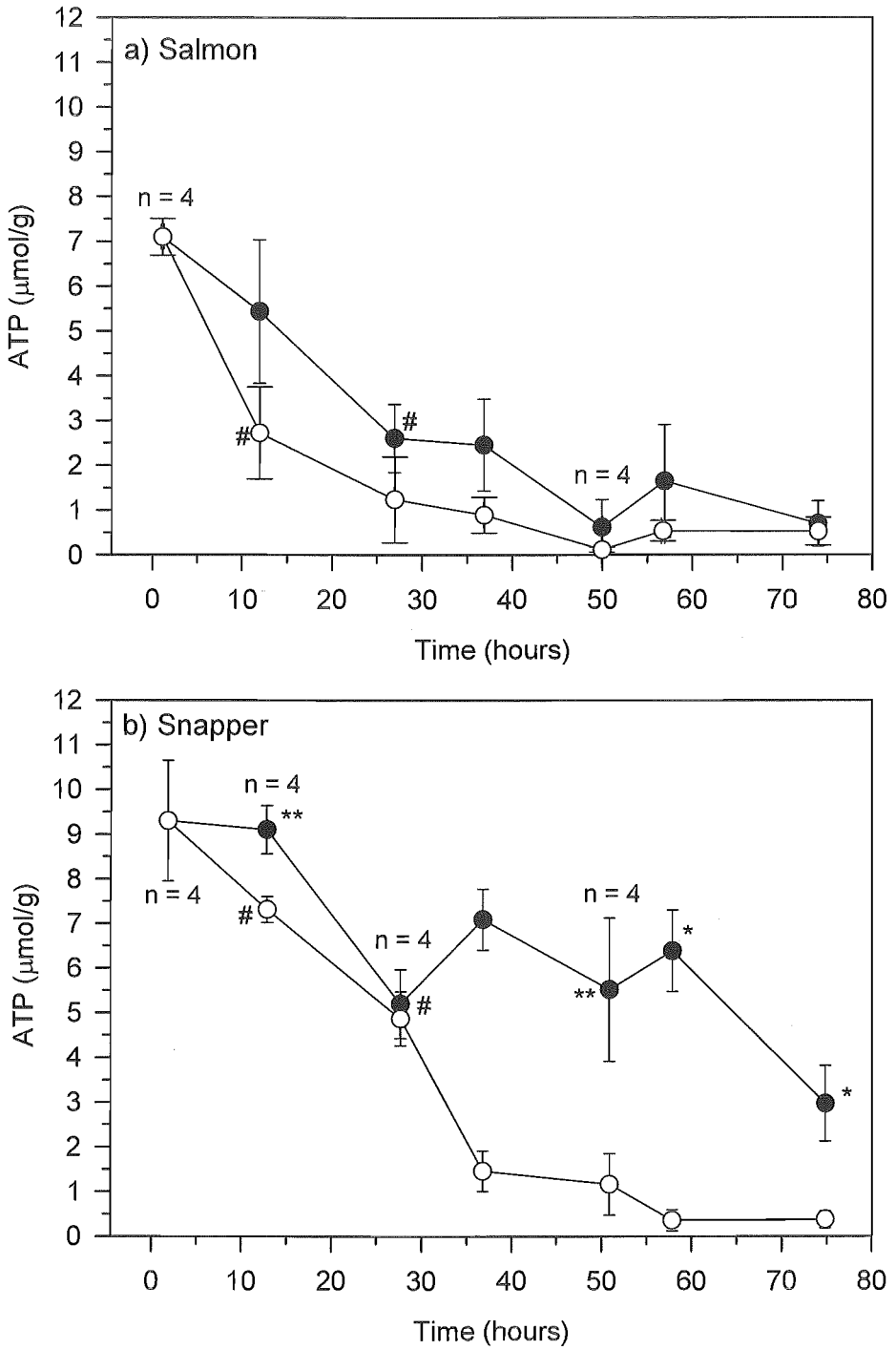

Figure 5.4: *Post-mortem [ATP] of the epaxial white muscle of a) chinook salmon and b) snapper stored under hyperbaric (●), or normobaric (○) conditions. Storage details as for Fig. 5.1. Values are the mean ± SEM, n = 5 unless otherwise stated. * indicates a significant difference (P<0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. ** Significantly different from the corresponding normobaric value at the same sampling time (Sign test P = 0.06 as n = 4). # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P<0.05).*

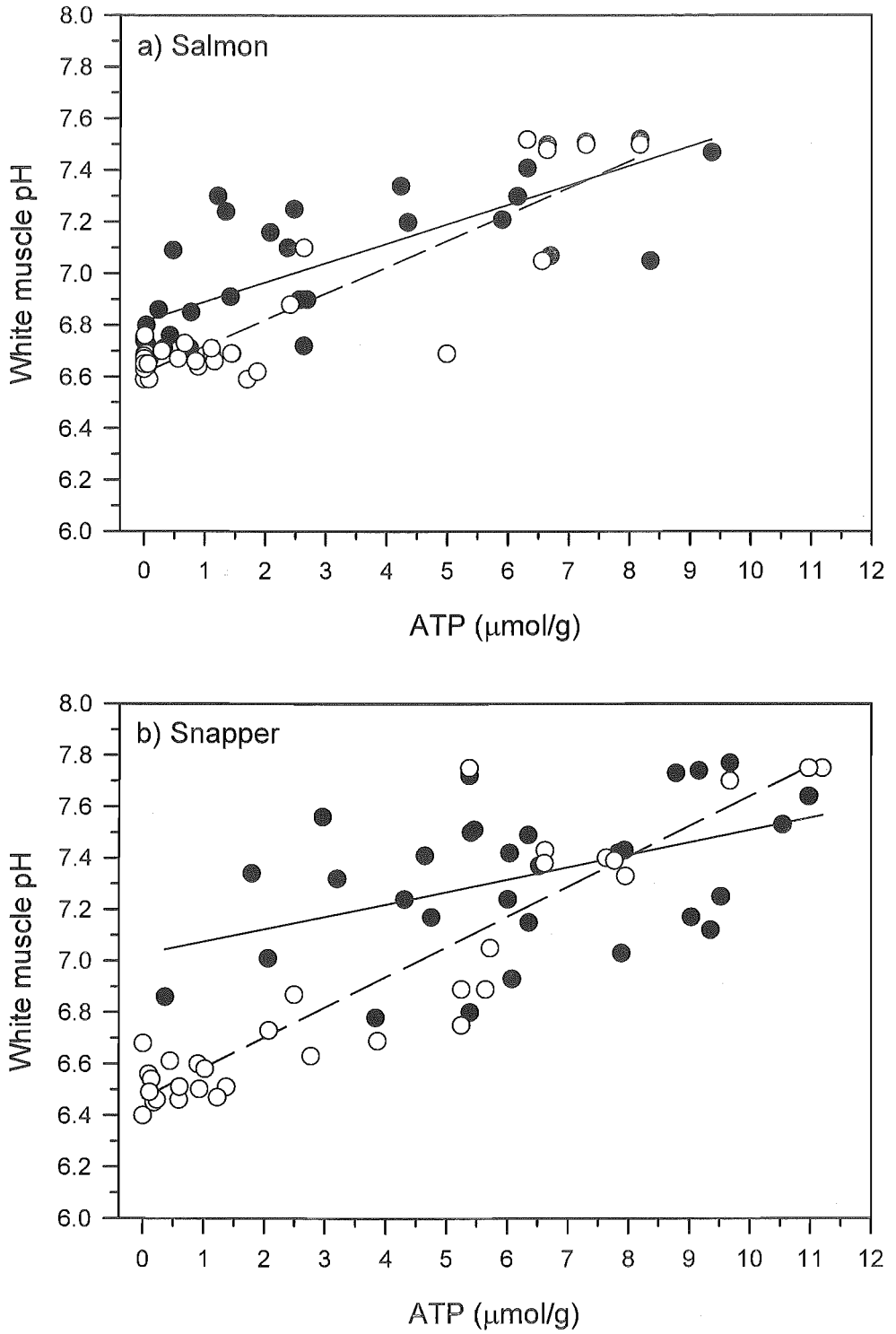

Figure 5.5: *Relationship between post-mortem cut-surface pH and [ATP] from the epaxial white muscle of a) salmon, and b) snapper. The pH/[ATP] relationship of hyperbaric salmon white muscle (●, solid line) is described by the linear regression equation* $y = 0.075x + 6.81$, $r^2 = 0.61$; *for normobaric white muscle (○, dashed line)* $y = 0.102x + 6.61$, $r^2 = 0.79$. *The pH/[ATP] relationship of hyperbaric snapper white muscle (●, solid line) is described by the linear regression equation* $y = 0.048x + 7.02$, $r^2 = 0.22$; *for normobaric white muscle (○, dashed line)* $y = 0.117x + 6.47$, $r^2 = 0.85$.

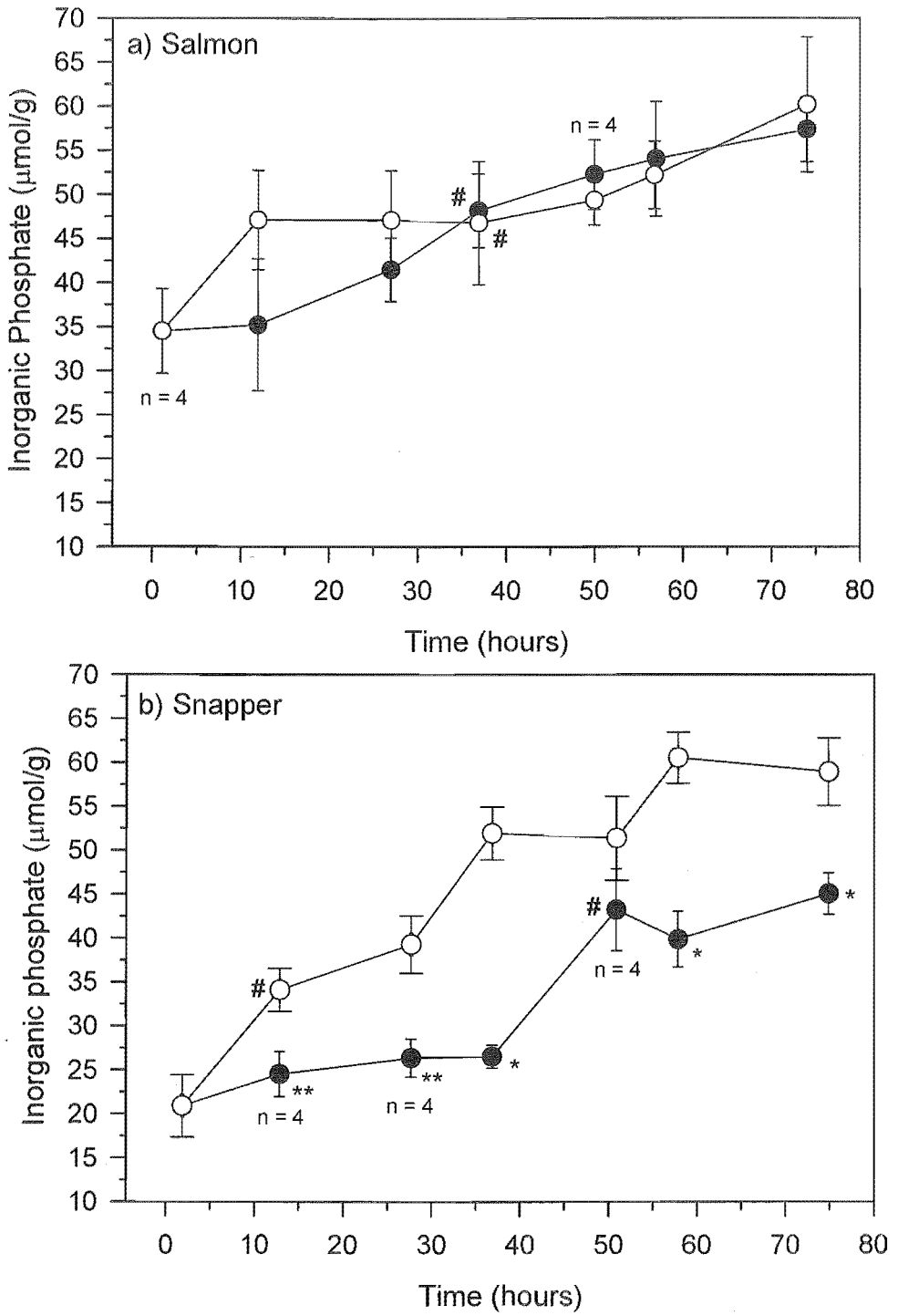

Figure 5.6: *Post-mortem [P_i] content of the epaxial white muscle of a) chinook salmon and b) snapper stored under hyperbaric (●), or normobaric (○) conditions. Storage details as for Fig. 5.1. Values are the mean ± SEM, n = 5 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. ** Significantly different from the corresponding normobaric value at the same sampling time (Sign test P = 0.06 as n = 4). # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

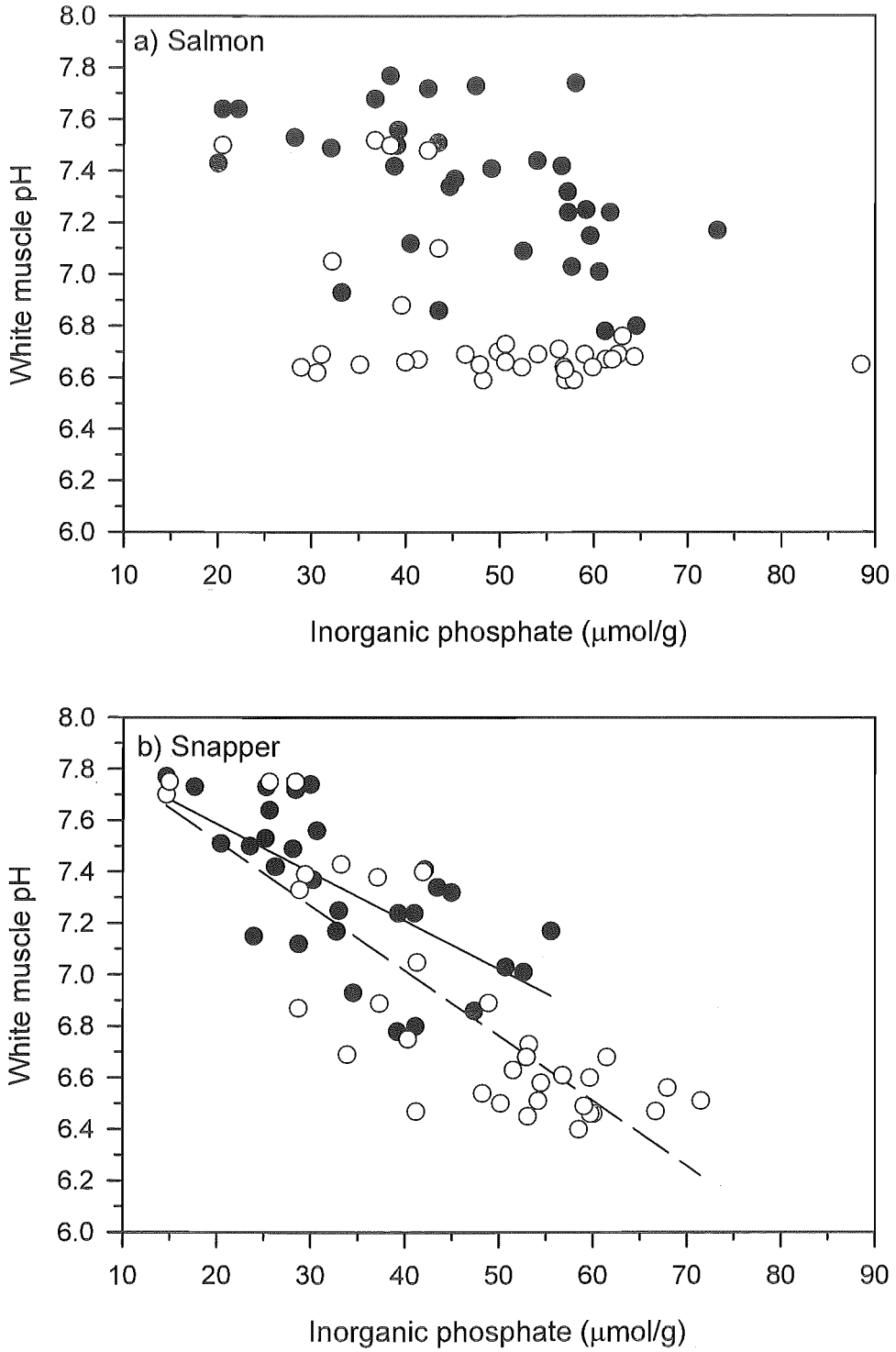

Figure 5.7: *Relationship between post-mortem cut-surface pH and $[P_i]$ from the epaxial white muscle of a) salmon, and b) snapper. The pH/$[P_i]$ relationship in hyperbaric salmon white muscle (●) and normobaric white muscle (○) was poor and therefore no regression lines have been fitted to the data. The pH/$[P_i]$ relationship of hyperbaric snapper white muscle (●, solid line) is described by the linear regression equation $y = -0.019x + 7.96$, $r^2 = 0.48$; for normobaric white muscle (○, dashed line) $y = -0.025x + 8.03$, $r^2 = 0.70$.*

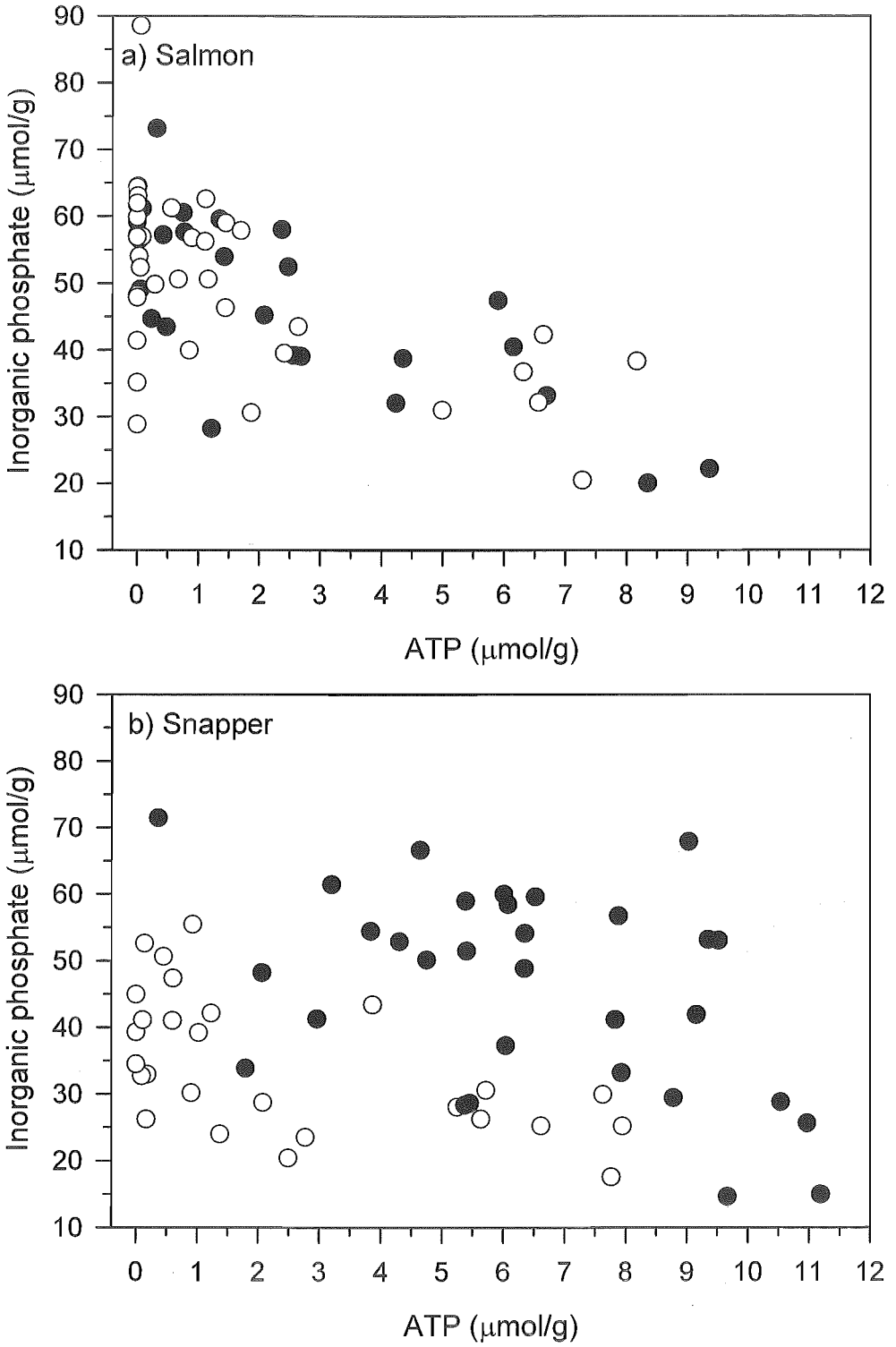

Figure 5.8: *Relationship between post-mortem [P_i] and [ATP] in the epaxial white muscle of a) salmon and b) snapper stored under hyperbaric (●) or normobaric (○) conditions*

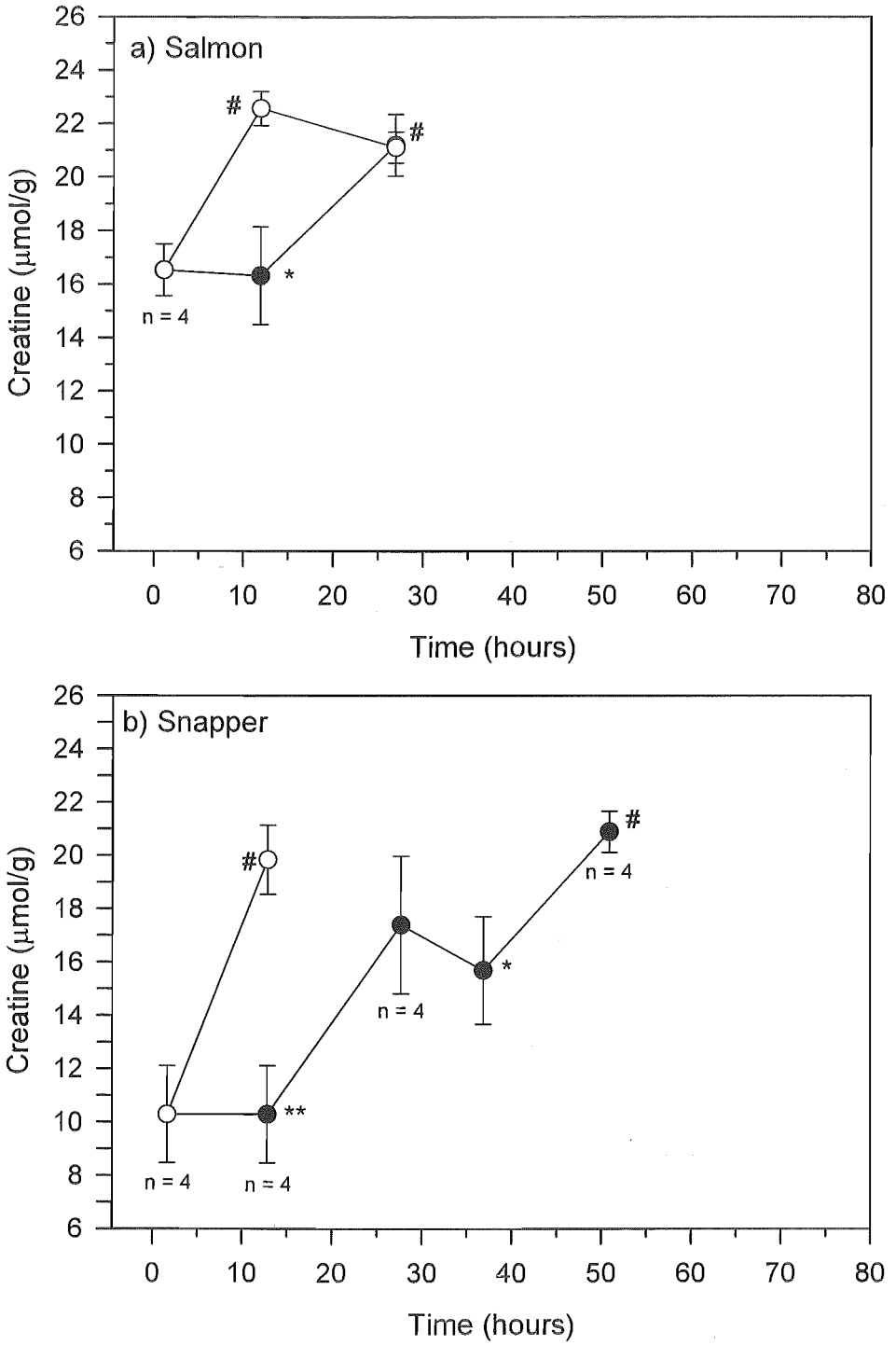

Figure 5.9: *Post-mortem creatine content of the epaxial white muscle of a) chinook salmon and b) snapper stored under hyperbaric (●), or normobaric (○) conditions. Storage details as for Fig. 5.1. Values are the mean ± SEM, n = 5 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. ** Significantly different from the corresponding normobaric value at the same sampling time (Sign test P = 0.06 as n = 4). # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

5.5 DISCUSSION

5.5.1 Methodology

There was a significant difference in the time it took snapper to reach anaesthesia compared with the salmon and mullet. The snapper took almost 30 min longer to reach a similar state of anaesthesia. This difference reinforced the need for all three species to be anaesthetised slowly and calmly, as the main objective of the experiments was to compare and contrast the PM changes of metabolites in WM from rested fish. The difference in anaesthetisation times was not believed to have significantly affected the outcome of the experiment but the different physiological response to anaesthesia may be another facet of species differences observed post-mortem.

The small size of the fish fillets limited how long the PM storage trials could run for as the WM was serially sampled. As discussed in Chapter 2 (White muscle pH measurement) two studies have shown that there are few positional sampling artefacts in [ATP] and enzyme activities in fish WM (Lowe 1992, Somero & Childress 1980). But it is unclear if the PM WM metabolism was altered in any way when the fillet became smaller and smaller, particularly at the later sampling times.

5.5.2 Pre-storage condition

Physical condition and blood measurements

Ideally, when attempting to make comparisons between species the animals used for experimentation should be of similar size and condition. However, in practice this is often difficult to achieve. The populations of fish that the snapper and mullet were sampled from were well conformed (good muscle tone in the belly) and in good condition. These fish were mainly fed on the alginate-bound moist diet. In contrast, the salmon used in the current study were not well conformed (muscle tone in the belly was poor) and did not put on weight at the expected rate during rearing (compared with previous populations of salmon smelt, A.R. Jerrett unpublished data). Salmon were primarily fed on a high lipid content pellet diet, had problems with scale loss on handling and developed a phenomenon called G-DAS (gastric-dilation air sacculitus). This was later attributed to the commercial pellet diet used and more specifically a deficiency problem. Salmonid WM is normally relatively high in lipids (stored

primarily as triglycerides) but the salmon used in the current study had WM that appeared dry (not glistening like healthy salmon, personal observation), suggesting that the lipid content of the muscle was low. It was apparent from the salmons poor growth during rearing that they were unable to utilise the high lipid content in the diet and convert it to muscle. This resulted in fish with poor muscle growth, poor CF and poor HSI. This also suggested that glycogen stores could have been low in these fish (Love 1980). It is likely this impacted directly on the PM metabolism of the WM in these fish and will be discussed in a later section along with the implications of the deficiency problem linked with the diet. Unfortunately it was not until after these experiments were carried out the some of the discrepancies were investigated.

The mean blood pH of rested snapper (7.59 ± 0.08) was similar to that reported in the mullet in Chapter 4 (7.64 ± 0.04) and in tank-rested chinook salmon (7.64 ± 0.04 at 12.4 °C, Jerrett and others 2002a), but slightly lower than rainbow trout (7.88 ± 0.01 at 15 °C, Turner and others 1983). Resting blood lactate levels were low and also in agreement with reported values (rainbow trout 0-2 mmol/L, Milligan 1996; coho salmon 1.0 ± 0.1 mmol/L, Milligan & McDonald 1988). The mean blood glucose concentration was also low.

The lower blood pH in the salmon (7.44 ± 0.07) indicated that the fish may have experienced some peri-mortem exercise. This was not reflected in high blood lactate concentrations but the blood glucose concentration was slightly higher. It was possible that the salmon in the current study may have experienced a slight hypoxia during anaesthetisation, even though they took less time to reach anaesthesia than the snapper. The salmon may have reached a deeper level of anaesthesia than the snapper, enough for the salmon to become slightly hypoxic depressing the blood pH, but not so hypoxic as to promote accumulation of blood lactate.

Pre-storage white muscle pH and metabolite levels

The initial mean (± SEM) cut-surface pH of the rested chinook salmon WM (7.48 ± 0.02) was slightly higher than those reported for chinook salmon in previous studies using similar rested harvesting procedures (pH 7.38 ± 0.05, Jerrett et al. 1996; pH 7.35 ± 0.04; 1988). However, it was noted in these studies that some disturbance to the animals occurred during the harvesting procedure and it was assumed that the true

resting pH value was slightly higher. The pre-storage cut-surface pH in snapper (7.72 ± 0.02) was higher than the chinook salmon and was also slightly higher than that reported elsewhere for P. auratus (7.62 ± 0.06, Jerrett et al. 2002b; 7.58 ± 0.16, Law et al. 1997). This was attributed to conservative harvesting and handling procedures.

Resting lactate levels in the WM of salmon and snapper showed some variation to those reported in the literature for salmonids (0 to 11 μmol/g muscle mass, rainbow trout review by Milligan 1996; Dobson et al. 1987). The mean pre-storage lactate levels in salmon and snapper in the current study were somewhat higher (20.6 ± 2.3 and 16.5 ± 2.4 μmol/g muscle mass respectively). In other studies carried out by the author's laboratory, pre-storage WM lactate levels for tank-rested anaesthetised chinook salmon were reported to be 8.08 ± 0.61 μmol/g muscle mass (n = 5; A.R. Jerrett unpublished results) and 5.73 ± 0.55 μmol/g muscle mass with a cut-surface pH of 7.51 ± 0.05 (n = 5; Jerrett et al. 2000). In the present study the initial lactate concentration of 20.6 ± 2.3 μmol/g muscle mass in the salmon and an initial WM pH of 7.47 ± 0.02 indicated that the fish used for this experiment may not have been as fully rested prior to, or during anaesthesia even though the initial pH was similar to those reported in other studies. In the case of snapper it may be a species difference as a resting lactate value of 21.1 ± 1.8 μmol/g muscle mass has been reported for P. auratus (slightly higher than the current study due to animal handling differences; Lowe 1992). Jerrett et al. (2002b) reported resting levels of WM lactic acid as low as 4.5 ± 1.4 μmol/g muscle mass in P. auratus using similar anaesthetisation techniques as employed in the current study. However, these fish were much smaller (209.5 ± 0.04 g) than those in the current study (634.3 ± 52.6 g). Large rainbow trout have been shown to have higher muscle lactate concentrations and also produce lactate at a faster rate when chased to maximal activity (Somero & Childress 1980).

As discussed in Chapter 4 for mullet, pre-storage ATP levels were slightly higher in salmon and snapper than those reported for rainbow trout (5.74 μmol/g muscle mass, Thomas et al. 1999; 4.99 ± 0.26 μmol/g muscle mass, Dobson et al. 1987) and Atlantic salmon (4.51 μmol/g muscle mass, Thomas et al. 1999). However, a similar resting level of ATP found in snapper in this study (7.43 ± 0.74 μmol/g muscle mass) was recorded by Lowe (1992) also in snapper (7.16 ± 0.89 μmol/g muscle mass). Again, controlled anaesthesia and conservative handling are the most likely reason for the conservation of the ATP store in the WM.

Resting P_i values were slightly higher in snapper compared with rainbow trout (15.2 ± 0.6 μmol/g muscle mass, Dobson et al. 1987), however, the value recorded for salmon was considerably higher (34.5 ± 4.8 μmol/g muscle mass). This gave another indication that the salmon were not in their most rested physiological state pre-mortem.

Although there were problems with the free creatine assay (readings above 20 μmol/g muscle mass were not reliable), the mean resting levels in salmon and snapper were below this (16.5 ± 1.0 and 16.7 ± 2.5 μmol/g muscle mass, respectively), and therefore an assumption was made that these were correct. As described in Chapter 4, higher levels of free creatine (31.50 ± 0.87 μmol/g muscle mass) were reported by Dobson et al. (1987) in resting rainbow trout. However, the total amount of phosphocreatine (PCr) present in rainbow trout was thought to be 40 μmol/g muscle mass (from ^{31}P-NMR analysis), which is somewhat higher than reported in other species (20-30 μmol/g muscle mass, van den Thillart & van Raaij 1995). Assuming that ~30 μmol/g muscle mass of PCr was present in the WM of the three species in this study, then it is possible that approximately half the PCr was broken down to creatine prior to freeze-clamping. This is assuming that all the creatine is in the phosphorylated form when the fish was at rest.

As described in Chapter 4 for mullet the maintenance of the ATP potential and minimal disturbance to the acid/base balance in rested salmon and snapper WM was attributed to controlled anaesthesia and conservative handling.

5.5.3 Post-mortem white muscle metabolism

Although the condition of the salmon used in the current study was poor there were clear differences in PM WM metabolism between hyperbaric and normobaric storage conditions. Unfortunately the poor condition made it difficult to make interspecies comparisons with any degree of confidence. As a result of this the majority of comparisons will be made between the snapper and mullet.

Post-mortem white muscle pH profiles

PM storage of WM under hyperbaric, hyperoxic conditions significantly slowed the rate of WM acidification in both the salmon and snapper (Fig. 5.1a & b). Under normobaric conditions the apparent rate of acidification in salmon WM over the first 12 h of storage

was similar to that measured in normobaric mullet WM (Chapter 4). But the decline in pH in normobaric snapper WM was musch slower (Fig. 5.1a & b). Slower rates of decline in WM pH in rested chinook salmon of similar condition, but higher HSI, have been reported in other papers (ultimate pH reached after ~30 h, Jerrett et al. 1996; ultimate pH reached after ~30 h, Jerrett et al. 2000). In these studies the fish were stored at similar temperatures to this study but were stored in the round (whole fish) in RSW (refrigerated seawater). The size of these fish was also larger than those in the current study (~1000 g) but because WM anaerobic machinery scales positively with body size (discussed in Chapter 3) any effect of scaling (larger fish, lower metabolic rate) should have been minimised. Storage of the whole fish in RSW may have cooled the WM faster but the small size of the fillets used in the current study should have ensured a fast rate of cooling.

A slightly faster decrease in WM pH has been reported in rested chinook salmon sampled in winter and stored at 4 °C (Jerrett et al. unpublished data). In this study the WM was stored as a fillet. Other studies have shown that excising the WM from the carcass accelerates PM changes (Jerrett et al. unpublished data). This may partially explain the faster rate of acidification in the normobaric salmon WM in the current study, but the poor condition of the salmon may also have played a significant role. This will be discussed in following sections.

PM storage under hyperbaric conditions resulted in slower rates of WM pH decline compared with normobaric storage, but there were also differences in apparent rates between the species studied. The acidification in hyperbaric mullet WM (Fig. 4.5) was only slightly slower than that in normobaric snapper WM (Fig. 5.1b). Hyperbaric snapper WM became acidified much slower than hyperbaric mullet WM. The decrease in pH in hyperbaric salmon WM was similar to that in the hyperbaric snapper but because the ultimate pH was higher (due to poor condition, discussed below) it is not possible to draw solid conclusions from the data.

Differences in ultimate white muscle pH

The ultimate pH of normobaric snapper WM was reached after 37 h storage (Fig 5.1b), whereas the normobaric mullet and salmon WM reached their ultimate pH after only 27 h (Figs. 5.1a and Fig. 4.5, respectively). These differences in the time it took the WM

to reach its ultimate pH gave an initial indication that there were species differences with regard to the rate of PM metabolism. But it was the value of the ultimate pH that alerted the author to the possible interference poor physical condition could have had on PM metabolism in the salmon WM.

Normobaric mullet WM reached an ultimate pH of ~6.4 during PM storage and in snapper the pH reached was ~6.55. These ultimate pHs were in agreement with those measured in other PM experiments in mullet and snapper carried out in the author's laboratory (A.R. Jerrett, unpublished data). In the salmon WM the ultimate pH was even higher at ~6.65. Bate-Smith & Bendall (1949) studied factors determining the ultimate pH of rabbit muscle and found that the level of feeding and the degree of fatigue prior to death were the primary determinants of ultimate pH. They found that in starved rabbits with no exercise prior to death the ultimate muscle pH was higher than in rested, well fed rabbits (pH 6.34 ± 0.13 compared with 5.90 ± 0.10). This phenomenon has also been reported in Atlantic cod (Gadus morhua, Love 1980) with high ultimate pHs attributed to low glycogen stores in starved fish. As peri-mortem fatigue was minimised in the current study the nutritional state would have been the main factor in determining the ultimate pH. Both the mullet and snapper had high mean HSI values (1.81 ± 0.19 and 2.93 ± 0.41, respectively), but the salmon had a low mean HSI (0.77 ± 0.04), indicating that the higher ultimate pH in the salmon was probably due to lower glycogen stores in the WM.

Fortunately there have been several PM studies carried out on chinook salmon with higher CFs and HSIs using similar rested harvesting methods to those used in the current study (see Section 5.5.3). Jerrett et al. (1996) found that rested chinook salmon WM reached an ultimate pH of ~6.3 after 40 h storage (compared with ~6.65 in the current study). The chinook salmon used in the Jerrett et al. study were much larger (1358 ± 56.4 g compared with 538.7 ± 28.9 g) but they also had higher HSIs (1.41 ± 0.06 compared with 0.77 ± 0.04 in the current study). This comparison again supported the hypothesis that the salmon used in the current study did not have very good glycogen stores. Although glycogen levels in the salmon and snapper WM were not measured, the peak concentration of lactate accumulated during PM storage gave a good indication that glycolytic substrate was limited. In the salmon WM lactate concentration during PM storage only reached ~65 μmol/g muscle mass (Fig. 5.3a) compared with ~80 μmol/g muscle mass in the snapper (Fig. 5.3b) and mullet (Fig.

4.7a). Consequently, with less lactate being produced the WM pH in the salmon did not reach the same levels as that measured in the snapper and mullet.

Post-mortem white muscle metabolite profiles

As stated above, there were clear differences in PM metabolism between WM stored under hyperbaric conditions and normobaric conditions in all three species studied. When the WM was stored under normobaric conditions there were immediate increases in lactate, P_i and creatine concentrations, along with a rapid depletion in ATP levels. Storage under hyperbaric conditions delayed these changes and maintained ATP levels for limited periods. But the magnitude of the delay, and the rate the changes occurred at, was different between the species.

Overall, snapper WM benefited the most from hyperbaric PM storage with regard to maintaining ATP levels and delaying the onset of anaerobic glycolysis. A delay of ~37 h was apparent in the snapper, with the mullet having a ~27 h delay and the salmon ~12 h. This delay in depletion of ATP stores in the WM was attributed to aerobic generation of ATP as it was in the yellow-eye mullet (Chapter 4). It was expected that the salmon WM would gain the most benefit from hyperbaric PM storage due to their high aerobic capacity. It was thought that because lipid levels are normally high in salmonid WM (at least 15 μmol/g, stored primarily as triglycerides; Moyes & West 1995) and oxygen diffusion through muscle would be faster (O_2 is substantially more soluble in non-polar hydrocarbons than aqueous solutions; Desaulniers et al. 1996) the salmon WM would have had access to oxygen before the snapper and been able to respire aerobically, and maintain ATP levels for longer. But in this case the hypothesis did not appear to hold true. The salmon used in the current study were in poor condition and possibly resembled starved fish in their WM composition (low lipid and glycogen, Love 1980). With a low WM lipid content any advantage the WM may have had regarding faster oxygen diffusion under hyperbaric storage conditions would have been lost. It is possible that the poor condition of the salmon may have lead to smaller WM fibre size and increased water content (Love 1980), therefore decreasing the diffusion distance. But even if the diffusion rate in the salmon WM was faster than in the snapper and mullet it was thought that the probable lack of metabolic substrate played the major role in the rapid PM changes.

Even though the salmon used in the current study were in poor condition the WM was still able to respire aerobically for a limited period during hyperbaric PM storage. The rate of lactate accumulation in the WM was also greatly reduced compared with normobaric storage. It is quite possible that WM from salmon in good physical condition would be able to respire aerobically for a longer period. Repeating this experiment with salmon in good condition would, therefore, be of some interest.

In terms of physical condition the mullet and snapper were quite similar and this was reflected in similar pre-storage concentrations of metabolites, and also peak WM lactate and P_i levels during PM storage. The apparent rates of accumulation and degradation were the slowest in the snapper WM for all the metabolites measured, including WM pH. The differences in the salmon pre-storage metabolites and the profiles during PM storage were more likely due to the condition of the fish rather a specific species difference. Although it is unfortunate that few comparisons between species could be made regarding the salmon, the differences in PM metabolite profiles are still of interest when comparing good condition and poor condition fish.

Relationships between post-mortem white muscle pH and metabolites

Some of the differences in the salmon WM were more apparent when relationships were made between the PM WM pH and the metabolites themselves. The relationship between PM WM pH and lactate concentration in chinook salmon and snapper was, in general, strong and similar to that in yellow-eye mullet (Chapter 4, Fig. 4.8a). However, in normobaric salmon WM the relationship was poor (Fig. 5.3a). This was due to a rapid increase in lactate early in PM storage, resulting in a rapid decrease in WM pH in the normobaric salmon WM. This resulted in a large "gap" in intermediate pH and lactate values and hence the poor correlation between these two measurements. The pH/[lactate] relationship illustrated the large buffering capacity of the WM (minimal acidification during initial PM lactate accumulation) and was most pronounced in the hyperbaric snapper WM (Fig. 5.3b). Unfortunately the buffering capacity was not measured in the salmon and snapper WM, but it may not be surprising that the normobaric salmon WM probably had a poorer buffering capacity compared with snapper and mullet. Specific amino-acids, such as histidine, anserine and carnosine (histidine related compounds, HRCs), add to the buffering capacity of the WM. Salmonids have been reported as having quite high levels of free L-histidine in

their WM (~5 μmol/g muscle mass, Abe 2000) along with high concentrations of anserine (24.5 μmol/g muscle mass, Abe 1995). These compounds compensate for the salmonids high capacity for anaerobic glycolysis during repeated burst swimming. But when fish are starved the lipid reserves are mobilised first, followed by WM protein, and lastly glycogen (Love 1980; Navarro & Gutiérrez 1995). If the salmon had started to mobilise their WM protein due to their inadequate diet it is not unreasonable to suggest that the proteins associated with buffering capacity were also being depleted resulting in a reduced buffering capacity in the salmon WM. If the salmon had been in good physical condition the buffering capacity of the WM may have been similar or even greater than the snapper WM.

The relationship between WM pH and [ATP] in salmon and snapper was not as strong as observed in mullet. The pH/[ATP] relationship in normobaric snapper WM was more typical of the relationship seen in the mullet (Chapter 4, Fig. 4.9). As [ATP] decreased there was a corresponding decrease in WM pH (Fig. 5.5b). In normobaric salmon WM the rapid depletion of WM [ATP] and pH meant that the majority of ATP levels measured corresponded with the ultimate pH (Fig. 5.5a). As a result of this little information could be gained from this tissue regarding the PM WM pH/[ATP] relationship.

In both hyperbaric salmon and snapper WM, relatively high WM pHs corresponded with low [ATP] (Fig. 5.5a & b, from mullet would expect low pH/low [ATP]; Chapter 4 Fig. 4.9). The hyperbaric snapper WM had not reached its ultimate pH at the end of the storage period, possibly indicating that the maintenance of ATP levels in this tissue was not associated with lactate production. In contrast, the hyperbaric salmon WM had reached its ultimate pH at the end of storage and lactate accumulation had occurred. In this case low [ATP] at relatively high WM pH was more likely due to limited glycolytic substrate reflected in the lower peak [lactate] in the salmon WM compared with snapper. Lower levels of lactate in the hyperbaric salmon WM was the most likely reason for the high WM pH/ low [ATP] relationship in this tissue.

The relationship between WM pH and WM [P_i] in normobaric and hyperbaric storage was relatively strong in the snapper (Fig. 5.7b) compared with the mullet (Fig 4.9). However, in salmon WM the relationship was not as good (Fig. 5.7a). There was

little variation in WM pH with varying [P_i] because the ultimate pH in the normobaric WM was reached very early in PM storage. It is possible that further P_i was produced due to the further breakdown of ATP→ADP→AMP, thus resulting in the large variation in WM P_i concentration around the ultimate pH (~6.6, Fig. 5.7a). Better resolution of the pH/[P_i] relationship was gained by storing the rested salmon WM under hyperbaric conditions (Fig. 5.7b).

The correlation between WM [P_i] and [ATP] showed that in the normobaric salmon WM the highest levels of P_i occurred once the ATP had been depleted (Fig. 5.8a). This pattern was not as pronounced in hyperbaric salmon WM, most likely due to the slower rate of ATP depletion during PM storage (Fig. 5.4a). In the snapper WM the correlation between WM [P_i] and [ATP] showed a large difference between normobaric and hyperbaric WM (Fig. 5.8b). The correlation in normobaric WM was more similar to that seen in salmon WM with hyperbaric snapper having relatively high levels of P_i coinciding with high levels of ATP. This suggested that ATP was being used, but the levels in the WM were being defended due to oxidative phosphorylation at the same time.

The strong relationship between PM WM pH and WM [lactate] in mullet, snapper and salmon and the relationship between WM pH and [ATP] over the physiological range measured showed that measurement of the cut-surface WM pH during PM storage was a simple but reliable predictor of WM lactate and ATP concentration in these three species. It has also been shown to be a reliable predictor of [lactate] in two other New Zealand fish species, hoki (Macruronus novaezelandiae, a deep sea species) and blue cod (Parapercis colias, a bottom-dwelling in-shore species, Jerrett et al. unpublished data).

Defence of white muscle ATP during post-mortem storage

WM ATP levels were defended for the longest PM period in both the normobaric and the hyperbaric snapper WM. As well as maintaining ATP levels there was very little acidification of the WM during hyperbaric PM storage compared with mullet and salmon. This suggested that in hyperbaric snapper WM either the rate of metabolism had been reduced and ATP was being consumed at a fraction of the normal rate (i.e. hypometabolism), or that ATP was being generated through aerobic

metabolism more successfully than in mullet and salmon. The latter possibility appears most likely due to the fact that species that are able to substantially decrease their resting metabolic rate tend to do so during periods of prolonged hypoxia and low temperature, e.g. overwintering in frogs and hibernation (Donohoe et al. 2000; St-Pierre et al. 2000). Snapper do not encounter such challenges and therefore are unlikely to possess the means to deal with them. However, it is possible that the reasoning for the prolonged defense of ATP in the PM WM was a combination of a lower innate metabolic rate (i.e. not strictly hypometabolism) and more successful aerobic generation of ATP. The snapper WM had an apparent slower lactate accumulation rate in both the normobaric and hyperbaric treatments compared with mullet and salmon (Figs 4.6 & 5.2). This suggested that their overall rate of metabolism may be slower than the mullet and salmon resulting in a reduced rate of ATP consumption. As discussed above, high levels of WM P_i were associated with high WM [ATP] suggesting that ATP was being broken down to ADP and AMP at a reasonable rate and not at hypometabolic levels. Therefore, it is probable that aerobic generation of ATP also played a major role in the defense of the WM ATP levels.

Availability of substrate for generation of ATP

If the WM of the salmon used in the current study was low in lipid due to the deficient diet then it is probable that the fish were also deficient in some essential free fatty acids (FFA). Some gross signs of this deficiency are fin rot, reduced growth rate, reduced feed efficiency and increased mortality (National Research Council 1993), consistent with observations made regarding the salmon used in the current study. As described in Chapter 4 it was possible that while the WM was able to respire aerobically under hyperbaric conditions FFAs may have been able to be utilised to produce ATP while sparing carbohydrate sources. This would not be possible in the salmon if they were deficient in FFAs. Rapid ATP depletion and lactate accumulation in WM stored under hyperbaric conditions supported this. If the WM was also low in glycogen, as suggested by the low HSI, this would have compounded the effect by severely limiting available substrate for ATP generation. In snapper it was possible that the maintenance of ATP stores for the first 37 h storage was through aerobic utilisation of FFAs. This was assuming that the good condition of the fish meant that they were not suffering a deficiency problem.

5.5.4 Difference in rate of post-mortem changes in different species

Overall, hyperbaric snapper WM appeared to gain the most benefit from storage under hyperbaric conditions compared with salmon and mullet. Although snapper are unlikely to have the ability to substantially depress their metabolic rate to low levels (discussed previously) the results suggested that the resting metabolic rate of snapper WM was probably lower than mullet or salmon WM. In snapper WM stored under normobaric conditions the ultimate pH was reached ~10 h later in PM storage than in the other species (Fig. 4.5 and Fig. 5.1). The slow rate of lactate accumulation in both hyperbaric and normobaric snapper WM (Fig. 5.2) also suggested that the actual rate of glycolysis in the snapper WM was slower, possibly due to reduced activities of key enzymes that regulate flux.

Snapper also have a different mode of locomotion compared with the salmon and mullet. Snapper use labriform swimming whereby the pectoral fins are used for locomotion. Salmon and mullet swim using subcarangiform locomotion, such that the posterior two-thirds of the body are used to propel the fish. As a result of this the snapper skeletal muscle is used relatively infrequently compared with salmon and mullet. It is probable that the infrequent use of the skeletal muscle in the snapper was then related to the slow rate of PM changes in the current study.

The differences in the rate of PM WM changes between the three species may also reflect how rapidly they can recover from burst exercise (A.R. Jerrett, pers. comm.). Salmon are a "high performance" fish (see Introduction) and are able to swim aerobically at high speeds and burst exercise often. Mullet are a surface dwelling fish, also required to burst exercise with relative frequency. Thus, both mullet and salmon have to recover quickly from exercise bursts to be ready for subsequent bursts. Snapper, on the other hand, need to burst exercise far less frequently and therefore do not need to recover at as fast a rate compared with the mullet and the salmon. It is possible that the PM rate of biochemical changes may be matched to the rate of recovery. On recovery from burst exercise it has been reported that the rate of lactate disappearance is positively correlated with pyruvate kinase (PK) activity (Moyes & West 1995). Bottom-dwelling species, such as starry flounder (Platichthys stellatus) had the slowest rate out of the species measured and skipjack tuna (Katsuwonus

pelamis) had the fastest. It could then be suggested that if a fish has a high PK activity (converts PEP to pyruvate, see Fig. 1.2) then the rate of lactate production during anaerobic glycolysis would also be high. As the snapper WM had the lowest rates of lactate accumulation it could be hypothesised that snapper may have had lower PK activity in the WM than salmon or mullet. Lactate disappearance in the WM after burst exercise has also been reported to be correlated with mitochondrial capacity, measured by citrate synthase activity (Moyes et al. 1993). Again, the slowest rates were measured in starry flounder and the fastest in skipjack tuna.

Mitochondrial respiration is mainly stimulated by an increase in P_i and also by low ATP/ADP ratios. It is possible that the pre-storage [P_i] of the WM was enough to stimulate mitochondrial respiration under hyperbaric hyperoxic conditions in all three species even though the ATP/ADP ratios would have been high. In snapper the mitochondrial capacity of the WM may have been lower than salmon or mullet but this may have been more supportive of mitochondrial function. In salmon and mullet hyperbaric WM the rate of mitochondrial respiration may have been too high, and eventually been detrimental to the function of the mitochondria. As described in Chapter 4, the mitochondria may have become damaged during PM hyperbaric storage due to accumulation of waste products from aerobic generation of ATP, such as H^+ and CO_2. Together with formation of reactive oxygen species in the hyperoxic environment these factors probably lead to the inhibition of mitochondrial respiration. If the snapper WM had a lower metabolic rate coupled with a lower mitochondrial capacity this combination could have reduced the rate of waste product accumulation and reactive oxygen species formation. Consequently, the period of aerobic ATP generation in snapper could continue for longer than in the salmon and mullet.

From the results of the current study and the lactate disappearance/enzyme correlations it could be expected that less active, bottom dwelling species may have the slowest rates of PM metabolism. Some species of flat-fish are known to retain most of the lactate generated during exercise in the WM where it is metabolised in situ (flounder, Platichthys stellatus, Milligan & McDonald 1988; turbot, Scophthalmus maximus, Maxine et al. 2000). Thus, their recovery rate after exercise is slower than in species that release a greater proportion of the generated lactate into the circulation. Overall, the tolerance for accumulated waste, the resting metabolic rate and "duty cycle" of the muscle all may contribute to the PM survival of rested tissue.

5.5.5 Questions to arise from these experiments

It was unfortunate that the salmon used in the current study were in such poor condition. The high probability that their PM metabolism was abnormal made it difficult to make inter-species comparisons. However, this misfortune raises some interesting questions. If the metabolism of the WM can be adversely modified by diet, can the diet be changed in such a way as to positively alter the metabolic machinery of the WM in order to extend the PM "life" of the WM?

Rested snapper WM gained the most benefit from PM storage under hyperbaric conditions out of all the three species investigated. But what is it about snapper WM that makes this occur? It is possible that the rate the WM can recover from burst exercise relates directly to the rate of PM metabolism (slow recovery/slow PM metabolism). If this is true then it is of interest to extend the study to those species that recover very slowly from burst exercise. It may also be a property of the ultrastructure of snapper WM. Rested snapper WM immediately after harvest is almost gel like in that it is translucent and pliable but not firm. Salmon muscle is very firm, dense and elastic, with mullet WM being similar to snapper but more firm. It would be of interest to determine if the structure of the snapper WM allows faster diffusion of oxygen into the tissue and also removal of waste products (i.e. CO_2) out of the muscle. Or is the difference due to the possible lower mitochondrial capacity of the snapper WM allowing the mitochondria to respire for longer during PM storage due to a reduced rate of waste product and reactive oxygen species formation?

The questions that arose in Chapter 4 regarding cessation of aerobic ATP generation in the mullet WM during PM storage under hyperbaric conditions also apply to the salmon and snapper WM. The most probable reason for the switch to anaerobic glycolysis during PM storage was the effects that waste products of aerobic metabolism, such as H^+ and CO_2, and high levels of oxygen may have on the mitochondria: the site of oxidative phosphorylation. The following chapter attempts to determine what affect (if any) these factors have on mitochondrial function in vitro.

5.6 SUMMARY

Hyperbaric PM storage of rested snapper and salmon WM delayed the depletion of ATP and accumulation of lactate, P_i and creatine, compared with normobaric PM storage, as was reported for yellow-eye mullet (Chapter 4). However, the magnitude of the delay and the rate of subsequent metabolic changes was different between all three species. The snapper WM gained the most benefit from hyperbaric storage with ATP levels being maintained for ~37 h. In the mullet WM ATP was maintained for ~27 h and in salmon only ~12 h. It was hypothesised that salmon would gain the most benefit from hyperbaric PM storage due to their high aerobic capacity and high lipid content (faster oxygen diffusion). However, the salmon were in poor physical condition and their WM possibly resembled that seen reported for starved fish (low lipid, low glycogen and possible degradation of protein). This had a significant impact on the PM metabolism of the salmon WM. The ultimate pH of the salmon WM was higher than the snapper and mullet and it is likely that the lack of substrate (glycogen for anaerobic ATP generation and FFAs for aerobic ATP generation) limited PM metabolism. Even though the salmon WM was in poor condition, storage under hyperbaric conditions still reduced the apparent rate of lactate accumulation (even lower than in hyperbaric mullet WM) compared with the normobaric WM.

It was uncertain why rested snapper WM benefited so much more from hyperbaric PM storage, however, it was hypothesised that the rate of recovery of WM from burst exercise may mirror the rate of PM change. It is possible that snapper had a lower mitochondrial capacity and this may be an advantage for extended aerobic generation of ATP during hyperbaric PM storage. The tolerance for accumulated waste, the resting metabolic rate and "duty cycle" of the muscle all may contribute to the PM survival of rested tissue.

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CHAPTER 6

Energy production in the rested white muscle: factors affecting in vitro mitochondrial respiration and viability during post-mortem storage

6.1 ABSTRACT

The objective of this study was to evaluate the effects of two potential stressors (pH and CO_2) on in vitro mitochondrial function in the WM of the yellow-eye mullet. PM storage of the WM under hyperbaric conditions resulted in a period of aerobic generation of ATP followed by a possible/apparent switch to anaerobic glycolysis. Another objective was to investigate if the mitochondria cease to respire aerobically and if they do, at what time during PM storage. Mitochondria were extracted from the WM and their respiratory performance was assessed using pyruvate. The ability of mitochondria to respire at similar pH levels experienced by the cell during PM storage (pH 6.0 to 7.5) was assessed. The significance of CO_2 build up in the cell was also assessed (1 to 15% CO_2 in the incubation medium). Mitochondria were also extracted from the WM at various times during PM storage under hyperbaric conditions (620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%) and normobaric conditions (normobaric pressure with humidified oxygen flow set to 50 mL/min ± 1.25%) to determine when the mitochondria become poorly coupled. The results showed that extracted mitochondria were only slightly inhibited by pH and CO_2 levels similar to those found in the cell during PM storage and were never fully inhibited. However, a combination of high CO_2 and low pH resulted in poorer mitochondrial respiration than low pH alone. When WM was stored under normobaric conditions the mitochondria became poorly coupled after 24 h with hyperbaric WM mitochondria reaching similar levels after 30 h. The difference in cut-surface pH between the WM stored under normobaric and hyperbaric conditions was much larger than the difference in respiratory coupling. It is suggested that even though the WM was able to respire aerobically during hyperbaric, hyperoxic storage the storage conditions may not have been protective of the mitochondria. Normobaric storage conditions may have been

more protective of mitochondria respiratory function even though aerobic generation of ATP was not possible. It was possible that poor coupling may have been a possible explanation for the apparent switch to anaerobic glycolysis.

6.2 INTRODUCTION

The results from Chapters 4 & 5 showed that when rested ischemic WM was stored at its optimum temperature under hyperbaric, hyperoxic conditions the PM metabolic changes occurring in the WM (i.e. acidification, metabolite level changes) could be significantly slowed. The metabolic changes suggested that aerobic generation of ATP occurred during this treatment. The major question arising from those findings was, despite the fact that the ischemic WM was supplied with oxygen during PM storage, why did tissue lactate accumulate apparently indicating a switch from aerobic to anaerobic metabolism?

There were three likely scenarios as to why the WM could not indefinitely maintain ATP levels through aerobic generation of ATP during hyperbaric, hyperoxic PM storage.

i) the availability of oxygen to the WM may have been limited by diffusion and could not be supplied at a rate necessary for sufficient generation of ATP, i.e. inefficient delivery of oxygen to the tissue.

ii) Once the PCr stores had been depleted aerobic generation of ATP could not satisfy demand.

iii) Accumulation of waste products from aerobic generation of ATP, such as H^+ and CO_2, may have damaged the mitochondria and/or inhibited their function in some way.

Scenario i) can be challenged by some experimental results. Bubbles were observed, and high levels of oxygen were measured in the WM after ~27 h PM storage under hyperbaric conditions (Chapter 4). At the same time, lactate began to accumulate in the WM and ATP became depleted. This suggested that the WM was unable to use the oxygen after ~27 h. Over the first 27 h storage ATP levels were maintained in the WM suggesting that the energy demand was being fulfilled by oxidative phosphorylation due to the sufficient supply of oxygen to the WM.

It appeared that sufficient oxygen was being delivered to the mullet WM during hyperbaric, hyperoxic storage and that during the first ~12 h of PM storage the oxygen

was being consumed at a rate similar to supply. A question still remained as to how fast the oxygen was consumed by the mitochondria and what limited it. In the literature there are two popular hypotheses as to what limits the rate of oxygen consumption (V_{max}) in cells. The first is that the number of mitochondria in the WM is rate-limiting. The second hypothesis is that V_{max} is limited by oxygen transport. Because there are relatively few mitochondria in WM compared with oxidative muscle (i.e. red muscle), it seems plausible that the first hypothesis (number of mitochondria is rate-limiting) would be the major factor in limiting the rate of oxygen consumption. However, in studies investigating the rate of recovery in rainbow trout it has been found that there is a 28-fold excess in mitochondrial ATP-producing capacity over the added demand of lactate conversion to glycogen post-exercise (Moyes et al. 1992). Furthermore, it is unlikely that the WM capacity is ever achieved in vivo. WM mitochondrial respiration in vivo is suppressed at rest to less than 10% of its aerobic capacity by metabolites regulating oxidative phosphorylation, primarily the substrates and products of the mitochondrial ATPase (ATP, ADP, phosphate, Moyes et al. 1992). Thus, a low mitochondrial content in the WM does not appear to be the reason why the oxygen consumption rate would be limited.

The second hypothesis was concerned with oxygen delivery limiting the rate of oxygen consumption. In the previous experiments, it was assumed that oxygen was in excess in the WM stored under hyperbaric oxygen conditions due to the physical evidence (bubble appearance). This, in turn, suggested that at the time when [lactate] was increasing rapidly in the WM it was unlikely that mitochondrial oxygen consumption was limited by oxygen availability.

Scenario ii) suggested that PCr played a major role in maintaining the ATP levels in hyperbaric mullet WM during the first ~27 h storage. In this scenario when the PCr stores were depleted, aerobic generation of ATP alone could not satisfy demand. However, this scenario was weakened by the observation that creatine levels in the WM did not begin to rise in the WM until ATP depletion began and lactate started to accumulate. This, in turn, suggested that when the ATP levels were being maintained in the WM that it was most likely the result of oxidative phosphorylation alone, rather than aerobic generation of ATP being "topped up" by PCr.

Scenario iii) suggested that the waste products from aerobic generation of ATP may damage/inhibit mitochondrial function. One of the waste products of aerobic metabolism that has received little attention in PM tissue studies is carbon dioxide (CO_2). Uptake of oxygen by mitochondria dictates the production of CO_2. Its production occurs during oxidative phosphorylation when pyruvate is oxidised to acetyl CoA (2 molecules of CO_2 produced) and in the Citric Acid Cycle (4 CO_2's produced). Thus for every glucose molecule oxidised, 6 CO_2's are produced (Mathews & van Holde 1990). With no circulation to rid the cells of waste products in the ischemic WM preparation it is possible that the [CO_2] could increase to high levels not normally experienced in vivo. If CO_2 was not effectively removed at the cellular level, the resulting decrease in intracellular pH may have had a detrimental effect on mitochondrial function and hence V_{max} (Duerr & Hillman 1993). In Chapter 3 it was observed that stopping gas flow through the hyperbaric treatment chambers essentially counteracted the delay in acidification possible with hyperbaric hyperoxia and gas flushing. This suggested that elimination of waste products was an issue for the continuation of aerobic generation of ATP, lending weight to inhibition of mitochondrial respiration by CO_2 and other waste products of aerobic respiration.

From these results the focus of the investigation was directed toward the function of the mitochondria. The objective of the current study was to determine why the mitochondria apparently cease to function in the WM of mullet during storage under hyperbaric conditions. As acidification of the hyperbaric WM occurred even before the lactate concentration began to rise it was hypothesised that the drop in pH was inhibiting the oxidative phosphorylation in the mitochondria.

The mitochondrial respiration performance of mitochondria extracted from rested yellow-eye mullet WM was measured over a pH range similar to that measured in the PM muscle (6.0 to 7.5). To determine if the waste product of aerobic metabolism, CO_2, influenced oxidative phosphorylation, similar experiments to those carried out for pH were carried out with varying concentrations of CO_2 (0 to 15%) in the incubation media. The results from both these experiments suggested that neither CO_2 or low pH alone inhibited mitochondrial respiration. Further to these experiments, mitochondria were extracted from the WM at various stages during PM storage under either hyperbaric or normobaric conditions. The level of mitochondrial coupling was

measured at the different sampling times and this was related to the biochemical changes occurring in the WM at the time (Results in Chapter 4). These experiments indicated that mitochondrial coupling may be compromised in the hyperbaric, hyperoxic treatment resulting in a loss of aerobic ATP generation and increased reliance on anaerobic glycolysis.

6.3 MATERIALS AND METHODS

6.3.1 Experimental animals

Experiments investigating the effects of varying the incubation medium pH and CO_2 levels on in vitro WM mitochondria respiration were performed using yellow-eye mullet in 1998 and 2001. During the course of the pH trial, Nelson Haven seawater temperature ranged from 14.2 to 16.8 °C (mean = 15.6 ± 0.4 °C). During the CO_2 trial and the experiments to determine the K_m for pyruvate oxidation (kinetic trials) by the mitochondria the seawater temperature range was 18.8 to 19.6 °C (mean 19.2 ± 0.2 °C).

Approximately 40 h before the experimentation began the main population of yellow-eye mullet were anaesthetised with 25.0 ± 0.5 mg/L AQUI-S^{TM} Plus to facilitate the transfer of 50 mullet to five round plastic tanks (tank, water, air and feed as described in Chapter 2 for yellow-eye mullet). Fish were fully recovered (upright swimming and feeding) within an hour of transfer. Leftover fish from the main population were transferred to a 6.08 m^3 round tank supplied with sand-filtered seawater at 30 L/min and auxiliary aeration (typical dissolved oxygen content of 8.0 mg/L).

This transfer was performed twice during the course of experimentation, once before the pH trials (November 2000) and again before the CO_2 and kinetic trials (January 2001).

Seventeen mullet were used during the pH, CO_2 and kinetic trials. The mean weight (W) of the mullet was 290.9 ± 13.5 g (± SEM) and mean fork length (L) was 267 ± 6 mm. The mean condition factor (CF) of these animals expressed by the formula: W (g)/L^3 (cm) x 100 was 1.51 ± 0.04 (Love 1980). The mean hepatosomatic index (HSI) expressed by: liver weight (g)/W (g) x 100 was 4.00 ± 0.30 (Love 1980). In these experiments only one fish could be sampled at a time (i.e. each day) and therefore the other fish that had been anaesthetised in the tank had to be transferred to another tank for recovery. To avoid anaesthetising the same tank of fish every day for sampling 5 tanks of fish were used with a different tank of fish being anaesthetised each day an experiment was carried out.

Experiments to determine the effects of PM storage under normobaric, hyperoxic and hyperbaric, hyperoxic conditions on mitochondrial function were carried

out during October and November 2001. Yellow-eye mullet captured in January 2001 were used in the PM storage experiment over a period of 4 weeks. The seawater temperature ranged from 13.6 to 16.6 °C (mean = 15.3 ± 0.2 °C) during this period.

The mean weight of the 33 mullet used in the PM storage experiments was 94.3 ± 4.5 g and mean fork length was 203 ± 2 mm. The mean CF of these animals was 1.11 ± 0.02 and the mean HSI was 1.50 ± 0.07.

6.3.2 Fish sampling protocol

Fish were anaesthetised with AQUI-S™ Plus at a concentration of 30.0 ± 0.5 mg/L (see Chapter 2: Harvesting Method). The fish were sedated after 5 min but were not suitable for handling until ~25 min. At this point a fish was transferred into a 20 L container with 10 L of seawater and 30.0 ± 0.5 mg/L of AQUI-S™ Plus. Once anaesthetised a mixed venous blood sample was taken and pH, lactate and glucose measured (Chapter 2: Blood sampling and pH measurement).

6.3.3 Isolation of mitochondria from the white muscle

The fish were pithed immediately after the blood sample was taken and the WM was dissected from the carcass. Care was taken to remove all red muscle from the fillets. The WM was roughly chopped with a knife and then finely diced with a razor blade on a board that had been pre-chilled in the refrigerator. A 30 g portion of the diced tissue was placed in 50 mL of ice-cold isolation medium containing 140 mmol/L KCl; 10 mmol/L ethylenediaminetetra-acetic acid (EDTA); 5 mmol/L $MgCl_2$; 20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES); 0.5% bovine serum albumin (BSA); pH 7.5 at 20 °C (Moyes et al. 1989). The tissue was gently homogenised for 15 sec (Model: T50 on lowest speed; Ultra-turrax, IKA Laboratechnik, Staufen, Germany). The homogenate was then divided between four 50 mL centrifuge tubes that were then topped up with isolation medium. The diluted homogenate was centrifuged (Model: J21B; Beckman Instruments Inc., Palo Alto, CA, USA) at 1000 *g* for 5 min at 2 °C. The supernatant was filtered through 250 μm nylon mesh and centrifuged at 9000 *g* for 10 min at 2 °C. The resultant pellet was in two layers: a loose white layer and a yellow/brown firm layer (mitochondria). The loose layer was gently washed away using isolation medium (containing no BSA), leaving the mitochondria.

The mitochondria were re-suspended in three drops (~150 µL) of isolation medium (no BSA to aid in protein determination) and then pooled with the other pellets. This resulted in a suspension containing 1-2 mg mitochondrial protein/mL. Mitochondrial protein was determined using a protein assay based on the Bradford method (Cat. No. 500-0002; Bio-Rad Laboratories, Oakland, CA, USA) with the addition of 1 mol/L NaOH to break down the mitochondrial protein.

6.3.4 Respiration measurements

Determination of the rate of oxidation of pyruvate was made in a cylindrical glass respiration cell (OD 18 mm, ID 14 mm, length 61.5 mm, custom-made) using a Clarke-type oxygen electrode (Model: MI-730; Microelectrodes, Inc., Bedford, NH, USA) connected to a dissolved oxygen meter (Model: OM-4; Microelectrodes, Inc., Bedford, NH, USA). Measurements were recorded using a chart recorder ((Model: LR4100E; Yokogawa Electric Corporation, Tokyo, Japan). The glass cell was placed in a refrigerated water bath (Model: Y14; Grant, Cambridge, England) and suspended over a magnetic stirrer (Model: Micro; H+P Variomag Laborotechnik GmbH, Munich, Germany) connected to a speed controller (Model: Telemodul Electroniclührer; Variomag Laborotechnik GmbH, Munich, Germany). Between the bottom of the cell and the stirrer was a gap of ~5 mm to ensure the cell was surrounded by water and that any heat generated by the stirrer had a minimal effect on the contents of the cell. Enough mitochondrial suspension was added to the air saturated incubation medium (140 mmol/L KCl; 5 mmol/L Na_2HPO_4; 20 mmol/L HEPES; pH 7.5 at 20 °C; Moyes et al. 1989) in the respiration chamber to make a 0.1 mg/mL suspension. The volume of incubation medium and mitochondria was 2 mL and was stirred at a slow speed with a small stir bar (3 mm x 6 mm, Azlon, Bibby Sterilin Ltd., Staffordshire, England). Oxygen consumption was measured after the addition of 5 µL of 0.5 mol/L pyruvate (concentration in the 2 mL cell). A typical in vitro mitochondria respiration recording is shown in Fig. 6.1. The maximal rate (state III) was obtained by adding 5 µL of 0.02 mol/L ADP approximately 3-6 min later (Fig. 6.1). The state IV rate was obtained after all the ADP had been phosphorylated. The respiratory control ratio (RCR) was calculated from the ratio of the state III to the state IV rate. All measurements were carried out at the acclimation temperature of the fish. The oxygen electrode was

calibrated with air-saturated and nitrogen-saturated incubation medium. All chemicals were purchased from Sigma Chemical Co, St Louis, MO, USA.

6.3.5 Mitochondria K_m for pyruvate

The same method was used to examine the kinetics of pyruvate oxidation by intact mitochondria at the fishes acclimated temperature (20.0 ± 0.1 °C). Various concentrations of pyruvate were added to the preparation (0.02 – 12000 μM) producing varying respiration rates.

6.3.6 Effect of incubation medium pH on pyruvate oxidation

The incubation medium was adjusted to either pH 7.50 (standard), 7.00, 6.50, or 6.00 (measured to ± 0.01 pH units) using 5 N potassium hydroxide and 1 N hydrochloric acid. This was measured using a combined pH electrode (Model: pHC 2406, Radiometer, Copenhagen; calibrated as directed) connected to a pH/mV meter (PHH92 Lab Meter, Radiometer, Copenhagen, Denmark). The rate of oxidation of pyruvate by the mitochondria of each extract (one extract = one fish) was measured at all four incubation medium pHs. The order in which the incubation medium pHs were tested was randomised to compensate for the deterioration of the extract with time. This series of experiments was carried out at the acclimated temperature of the fish at the time (15.5 ± 0.1 °C).

6.3.7 Effect of incubation medium CO_2 content on pyruvate oxidation

The partial pressure of CO_2 in the incubation medium was adjusted to the desired value at the incubation temperature by bubbling various gas mixtures of 0% (0 torr), 2% (15 torr), 5% (37.5 torr), 10% (75 torr) and 15% (112.5 torr) CO_2 in air supplied by mass flow controllers (Models: 1179A, 0-10 SCCM and 0-200 SCCM; MKS Instruments, Andover, MA, USA) through the incubation medium until equilibrium was reached (~10 to 15 min). This was confirmed using a CO_2 electrode (Model: MI-720; Microelectrodes, Inc., Bedford, NH, USA), connected to a pH/mV meter (Model: PH-1; Microelectrodes, Inc., Bedford, NH, USA). The electrode was calibrated with gas mixes of 1, 5, and 10% (balance air).

To confirm that the mass-flow controller was delivering the correct amount of CO_2 a reference gas mixture was obtained (5.1 ± 0.1% CO_2, balance nitrogen; β standard, BOC Gases, Lower Hutt, New Zealand). An identical gas mix to the reference gas mixture (CO_2, balance nitrogen) was made using the mass-flow controllers and the accuracy of the controllers was confirmed by measuring the standard and the mass-flow controller mix with the CO_2 electrode.

The rate of oxidation of pyruvate by the mitochondria of each extract (one extract = one fish) was measured at all the incubation medium CO_2 partial pressures. Due to the deterioration of the extract with time the order in which the incubation medium CO_2 partial pressures were tested was randomised. This series of experiments was carried out 2 months after the pH experiments. The mitochondria were incubated at 20.0 ± 0.1 °C to compensate for the rise in the acclimated temperature of the fish.

6.3.8 Effect of post-mortem storage time on the ability to extract intact mitochondria

Mitochondria were extracted from the WM of rested mullet at various stages during PM storage. The standard storage protocols of Chapter 4 were used (normobaric pressure with humidified oxygen flow set to 50 mL/min ± 1.25% and hyperbaric conditions 620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25%). The amount of tissue used for each extraction was reduced from 30 g to 10 g to reduce the number of fish used in this experiment. This meant that only one fillet was required for each extraction, i.e. each fish provided two samples: one for normobaric conditions and one for hyperbaric conditions. The amount of isolation medium was reduced from 50 mL to 30 mL and the homogenisation time reduced from 15 sec to 7 sec to correspond with less tissue being used for the extraction. The homogenate was then split between two 50 mL centrifuge tubes and topped up with isolation medium. The rest of the extraction process remained the same. The changes to the extraction procedure did not appear to have an effect on the oxidation properties of the mitochondria.

A cut-surface pH measurement was taken (See Chapter 2: Section 2.6) prior to the mitochondria being extracted from the WM. Respiration measurements were made on each WM sample with the incubation medium adjusted to pH 7.5 and the RCR was calculated for each sample.

6.3.9 Statistical analysis

All pH values, rates and RCRs stated in the text are the mean ± standard error of the mean (SEM). Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows Version 6.00 (Copyright 1986-2000 SPSS Inc.) and Microsoft® Excel 2000.

6.4 RESULTS

6.4.1 Kinetics of pyruvate oxidation

The oxidation of pyruvate by mitochondria prepared from WM of rested yellow-eye mullet was described closely by Michaelis-Menten kinetics. The initial state III oxygen consumption (V_0) was plotted as a function of initial pyruvate concentration and is shown in Fig. 6.2 for mitochondria incubated at 20.0 ± 0.1 °C in air saturated medium (pH 7.5). The solid line was predicted by the Michaelis-Menten equation:

$$V_0 = \frac{V_{max}[P]}{K_m + [P]}$$

Where V_{max} is the maximum respiration rate; [P] is the concentration of pyruvate; and K_m is the concentration of pyruvate that will result in half the maximal respiration rate.

The dashed line is the regression equation (hyperbola, single rectangular, 3 parameter) represented by the equation:

$$y = y_o + \frac{a\,x}{b + x}$$

where $y_o = 16.51$; $a = 90.79$; and $b = 45.69$

V_{max} = 111.8 nmol O_2/min/mg protein, and K_m = 35.0 μmol/L for mitochondria from rested mullet WM.

6.4.2 Effect of incubation medium pH on pyruvate oxidation

There was no significant difference in RCR or V_{max} for mitochondria incubated at pH_e (extra-mitochondrial pH) 7.0 compared with the pH_e 7.5 control (Fig. 6.3). However, the difference was significant at pH_e 6.1 ($P < 0.05$) and close to being significant at pH_e 6.5 ($P = 0.057$). The decrease in RCR with decreasing pH_e was largely due to the decrease in the state III rate (V_{max}) (Fig. 6.3). However, there was a significant increase in the state IV rate as pH_e decreased, compounding the effect (Fig. 6.4).

The pH of the solution was measured when the mitochondria suspension was added to the incubation medium and then measured again after the mitochondria had gone through one addition of ADP to the incubation medium in the presence of

pyruvate. Incubation of mitochondria at pH_e 7.50 ± 0.00 and 7.08 ± 0.00 resulted in a slight drop in pH_e after respiration (not significant, Fig. 6.5). However, at pH_e 6.54 ± 0.01 the pH_e of the solution increased by 0.07 ± 0.01 pH units and at pH_e 6.10 ± 0.00 the increase was 0.19 ± 0.00 units.

Changing the pH of the incubation medium did not have any effect on the ADP/O ratio (Table 6.1). The ADP/O ratio is defined as the total amount of molecular oxygen required to phosphorylate all of the ADP added to the mitochondrial suspension. .

Table 6.1: *ADP/O ratio for mitochondria during pyruvate oxidation at various incubation medium pH.*

	pH 6.0	***pH 6.5***	***pH 7.0***	***pH 7.5***
ADP/O	*2.18 ± 0.05*	*2.22 ± 0.04*	*2.21 ± 0.04*	*2.28 ± 0.04*

Values are the mean ± SEM.

6.4.3 Effect of incubation medium CO_2 content on pyruvate oxidation

There were no differences in the RCR or V_{max} at 2, 5% and 10% P_{CO_2} (15, 37.5 and 75 torr, respectively) compared with the air control (P>0.05; Fig. 6.6 & 6.7 respectively). There was, however, a significant difference (P<0.05) in RCR at 15% (112.5 torr; Fig. 6.6) and V_{max} at P_{CO_2} levels of 10 and 15% (75 and 112.5 torr, respectively; Fig. 6.6 & 6.7). The change in RCR values with CO_2 concentration were compared with pH (Fig. 6.8). It can been seen that even though the incubation media pH changes with CO_2 concentration, the combination of the two stressors (pH and CO_2 concentration) did not greatly inhibit respiration. Only at a CO_2 content of 15% was there a significant difference in RCRs between the two trials at similar incubation medium pH.

6.4.4 Effect of post-mortem storage time on the ability to extract intact mitochondria

Mitochondria extracted from rested yellow-eye mullet WM were well coupled (high RCR) immediately prior to PM storage (Fig. 6.9). Mitochondria extracted from WM stored under normobaric conditions were well-coupled after 10 h storage and their RCRs were not different from the pre-storage level of 13.12 ± 1.15. After 10 h storage,

mitochondria extracted from WM stored under normobaric conditions became progressively less coupled, with an RCR of 2.41 ± 0.45 after 24 h storage (poorly coupled). WM held under hyperbaric conditions also had well coupled mitochondria after 10 h storage followed by a decline in coupling over the remaining storage period. The RCR had dropped to 2.96 ± 0.55 after 30 h storage (Fig. 6.9). After 24 h storage, mitochondria from WM stored under hyperbaric conditions were significantly better coupled (greater RCR) than mitochondria from WM stored under normobaric conditions. The ADP/O ratios during pyruvate oxidation are shown in Fig. 6.10 for the PM storage trial. Under normobaric conditions ADP/O steadily decreased during PM storage with the ratio being significantly lower than the pre-storage value after 24 h storage. The ADP/O for WM stored under hyperbaric conditions was more variable than in normobaric WM with the ratio after 30 h storage being significantly different from the pre-storage level. The correlation coefficient between RCR and ADP/O ratio was 0.60 in normobaric mitochondria but only 0.37 in hyperbaric preparations. The cut-surface pH of the WM during storage is shown in Fig. 6.11. The pH changes occurring during PM storage were consistent with other similar experiments (Fig. 6.11 compared with Chapter 4: Fig. 4.5). Throughout the experiment the RCR's of the mitochondria extracted from WM stored under hyperbaric conditions were lower at any given cut-surface pH than mitochondria extracted from WM stored under normobaric conditions (Fig. 6.12).

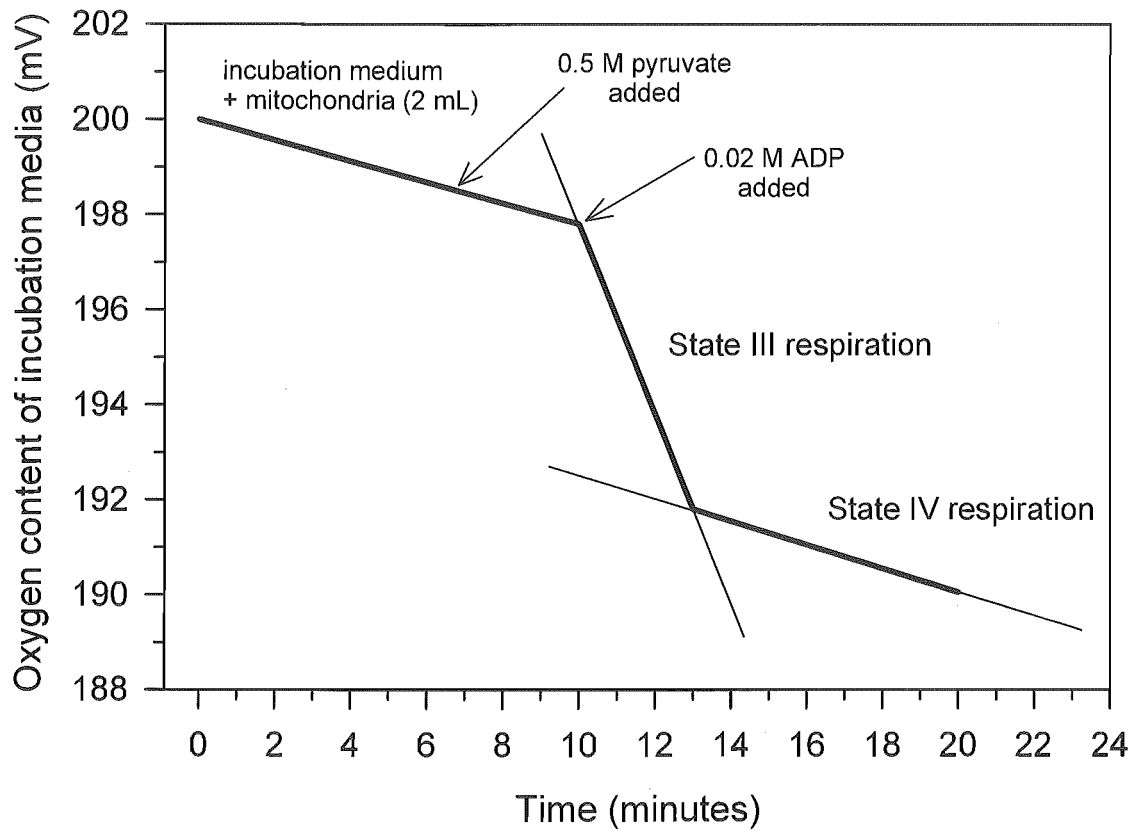

Figure 6.1: *A typical mitochondria respiration recording showing the transitions in respiration rate after the addition of substrate (pyruvate) and ADP. State III respiration occurs while there is ADP available to phosphorylate to ATP. State IV respiration occurs after all the ADP has been phosphorylated. The RCR (respiratory control ratio) is the ratio of the State III respiration rate to State IV. In this case the State III rate was 2 mV/min and the State IV was 0.25 mV/min, giving an RCR of 8.0.*

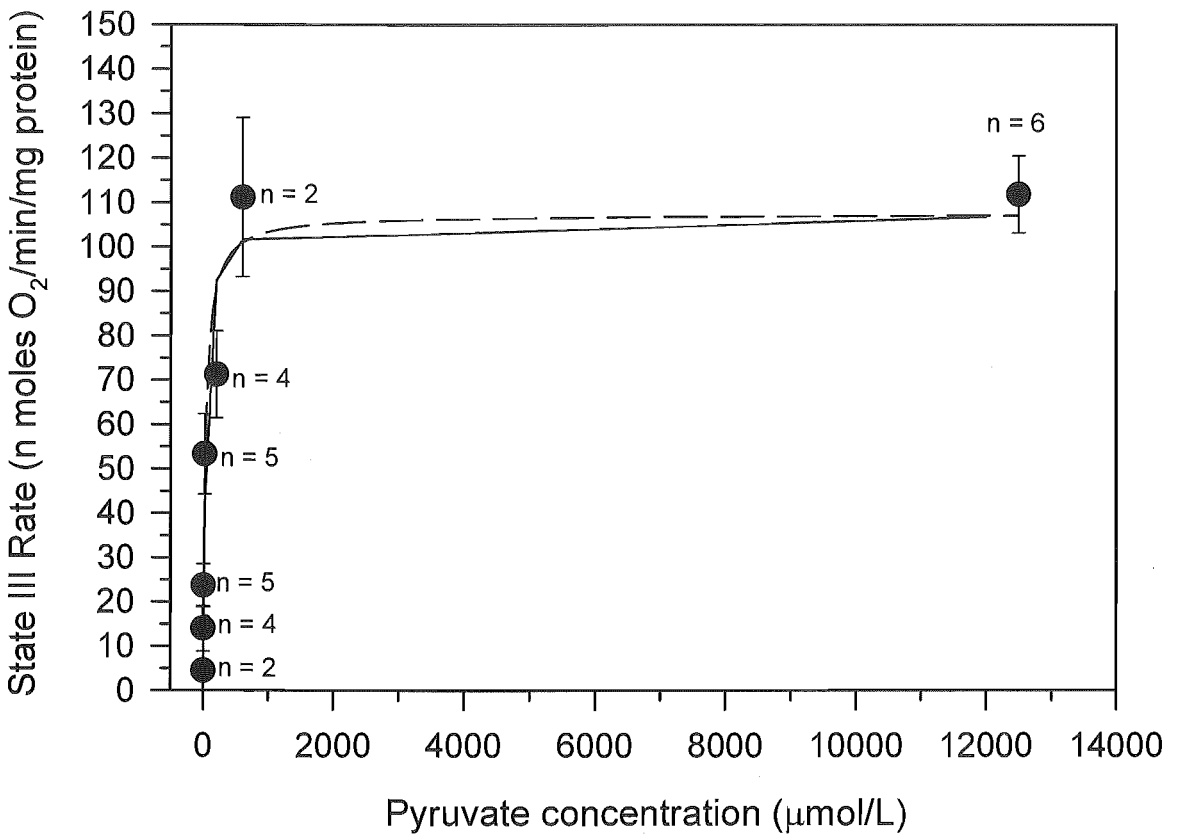

Figure 6.2: *Oxidation of pyruvate by yellow-eye mullet white muscle mitochondria as a function of substrate concentration. Preparations were incubated at 20.0 ± 0.1 °C, pH 7.5. Values are the mean ± SEM. Pyruvate concentration represents the initial concentration in the 2 mL volume. The solid line is the one predicted by the Michaelis-Menten equation. The dashed line is the one represented by the 3 parameter hyperbolic regression equation (see "Results" section).*

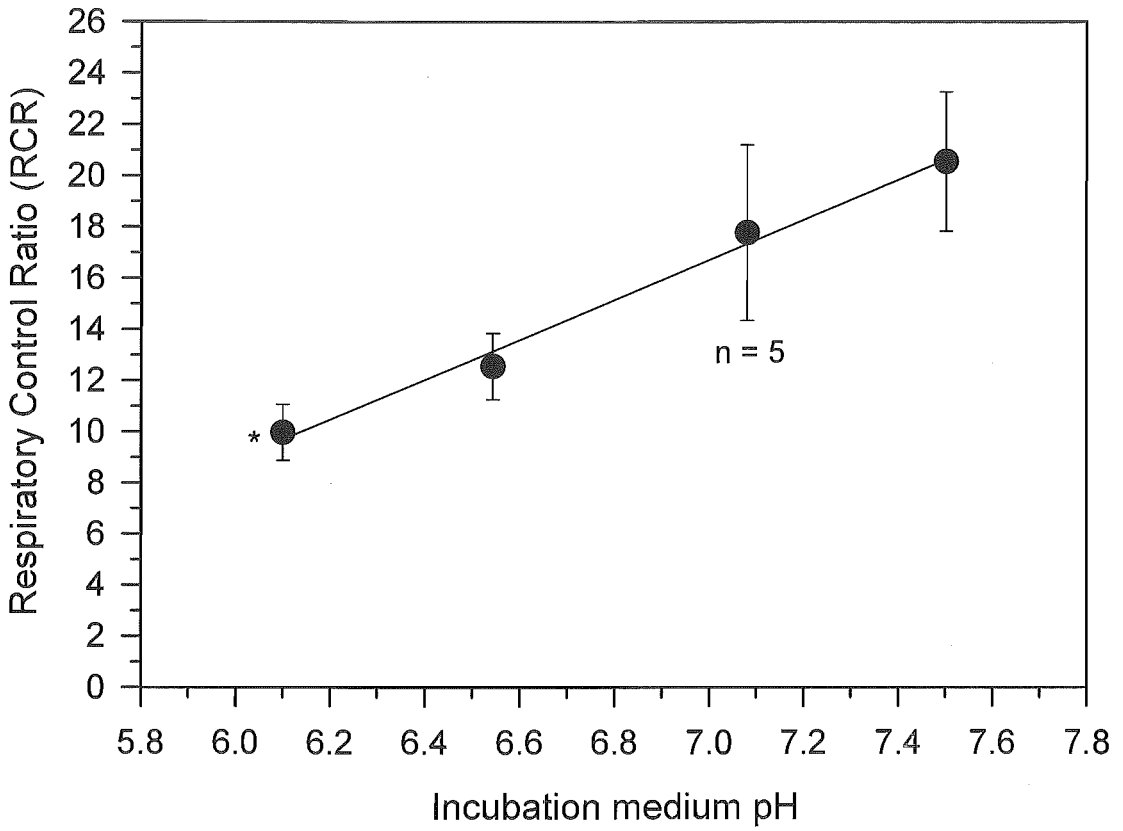

Figure 6.3: *Oxidation of pyruvate (measured by Respiratory Control Ratio, RCR) by yellow-eye mullet white muscle mitochondria as a function of incubation medium pH. Preparations were incubated at 20.0 ± 0.1 °C. Values are the mean ± SEM, n = 6, unless otherwise stated. * indicates a significant difference from the pH 7.5 value.*

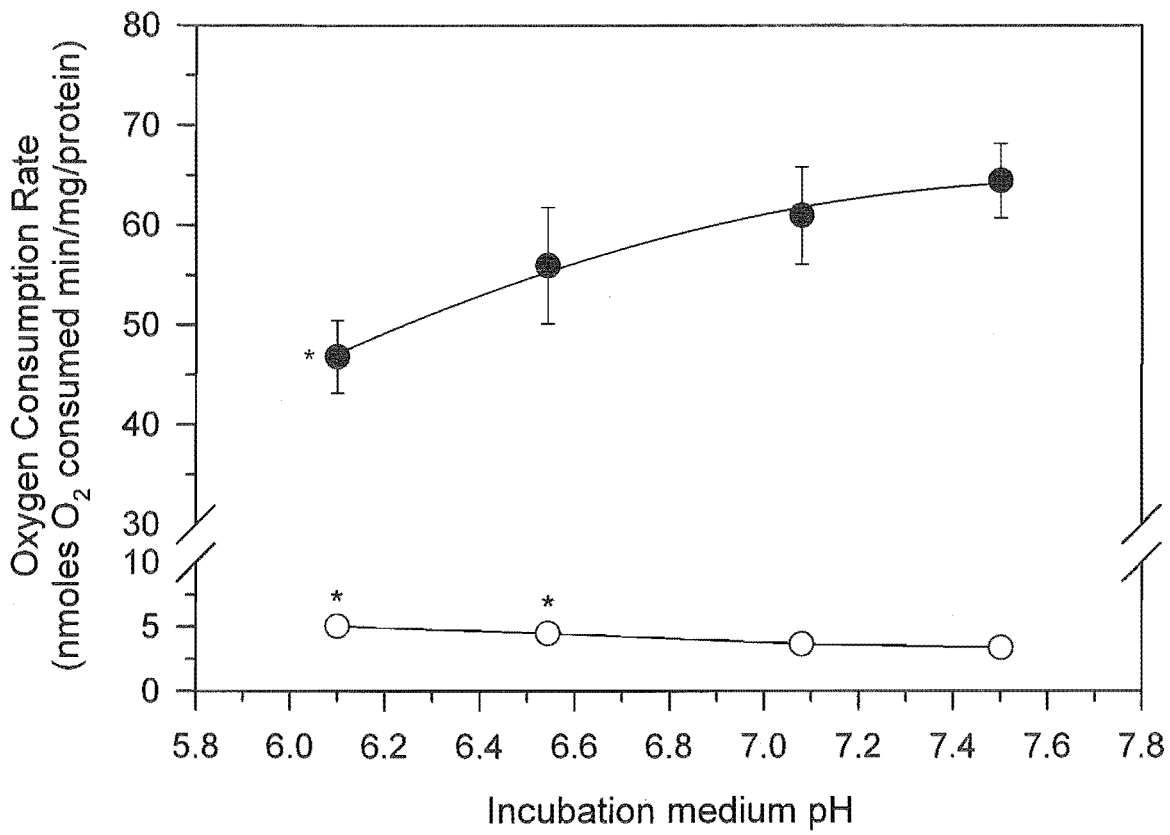

Figure 6.4: *Effect of incubation medium pH on State III (V_{max}) (•) and State IV (○) rates of respiration in yellow-eye mullet mitochondria. Preparations were incubated at 20.0 ± 0.1 °C. Values are the mean ± SEM, n = 6, unless otherwise stated. * indicates a significant difference from the pH 7.5 value.*

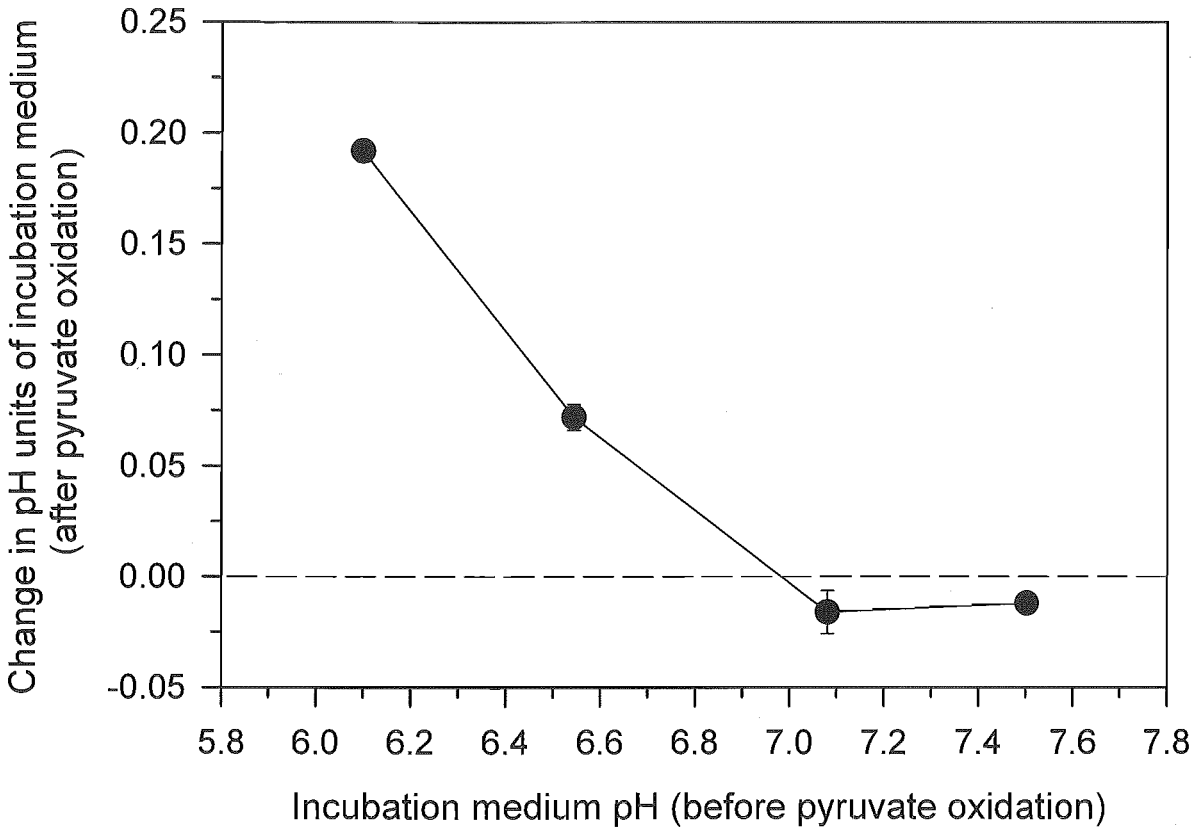

Figure 6.5: *Change in incubation medium pH after a period of State III pyruvate oxidation. Preparations were incubated at 20.0 ± 0.1 °C. Values are the mean ± SEM, n = 6, unless otherwise stated. * indicates a significant difference from the pH 7.5 value.*

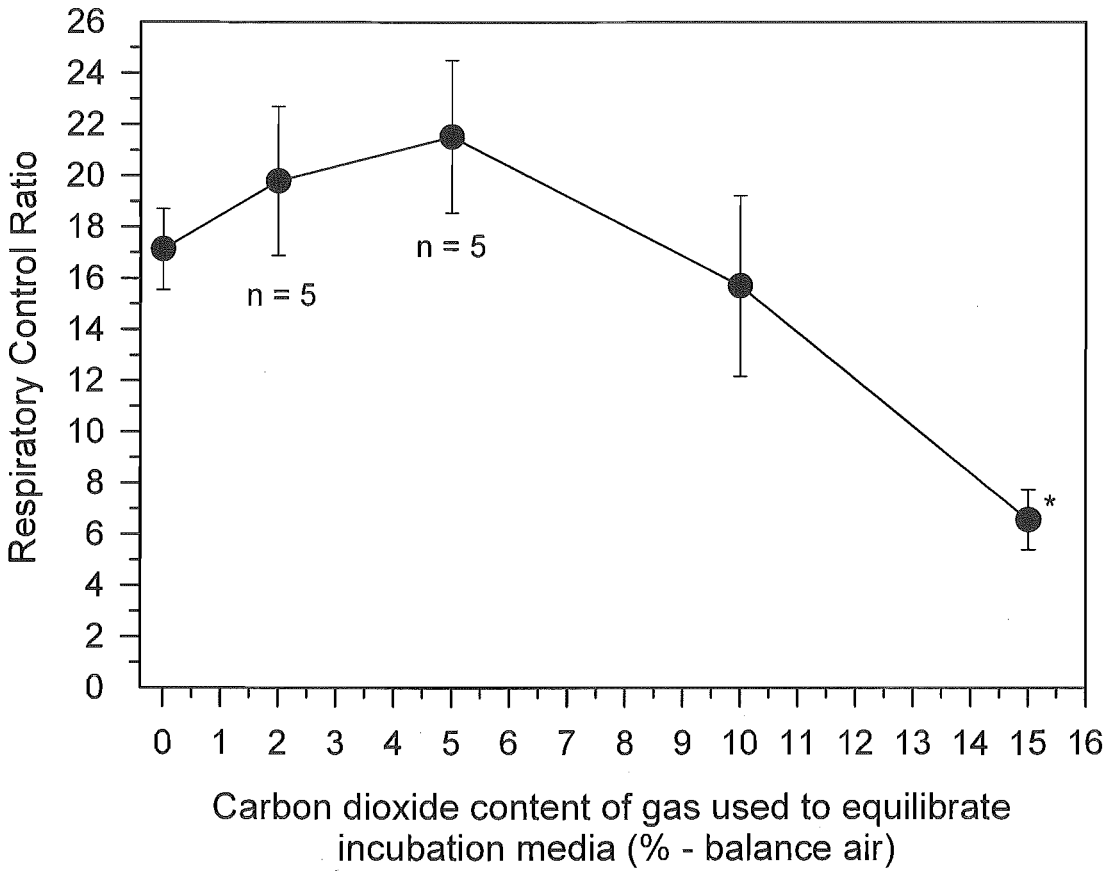

Figure 6.6: *Oxidation of pyruvate by yellow-eye mullet white muscle mitochondria as a function of incubation medium CO_2 concentration. Preparations were incubated at 20.0 ± 0.1 °C, pH 7.5. Values are the mean ± SEM, n = 6 unless otherwise stated. * indicates a significant difference from the control (0%) level.*

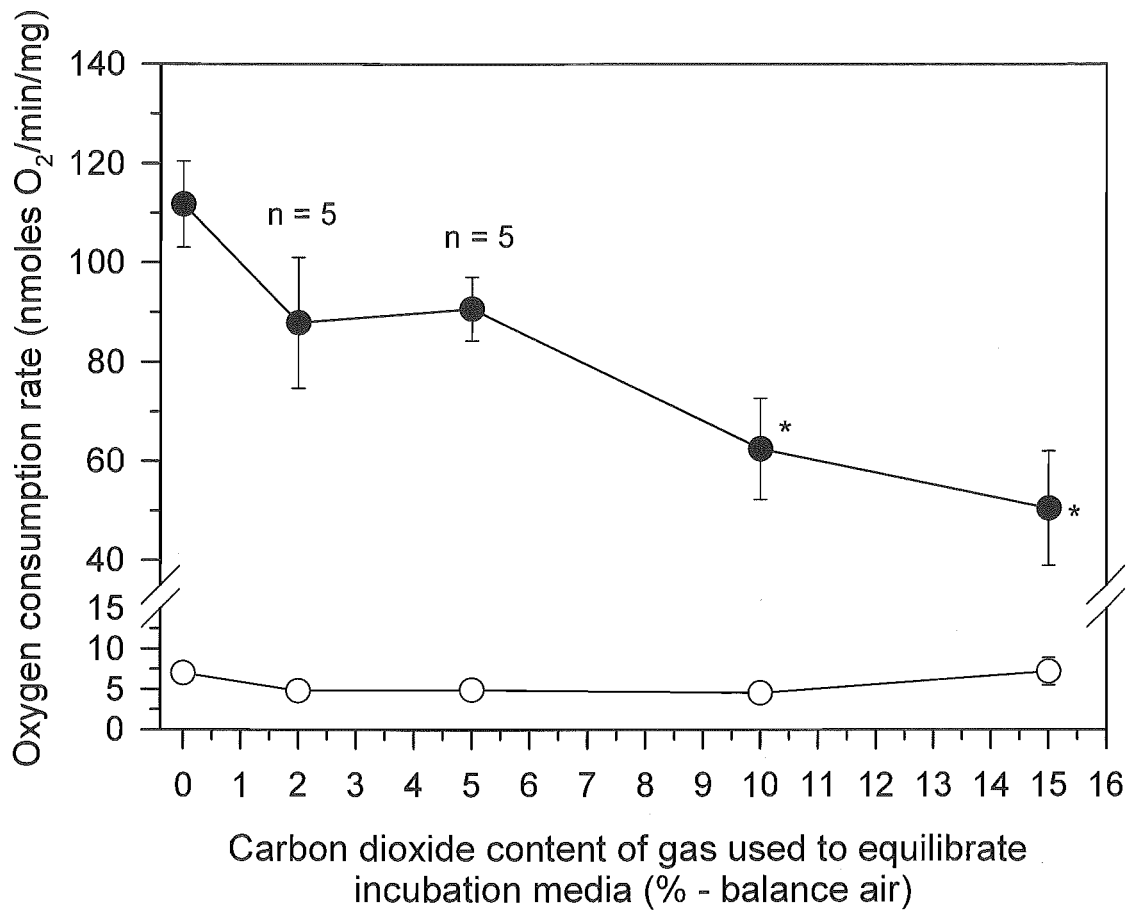

Figure 6.7: *Effect of incubation medium CO_2 concentration on State III (V_{max}) (●) and State IV (○) rates of respiration in mullet mitochondria. Preparations were incubated at 20.0 ± 0.1 °C. Values are the mean ± SEM, n = 6, unless otherwise stated. * indicates a significant difference from the control (0%).*

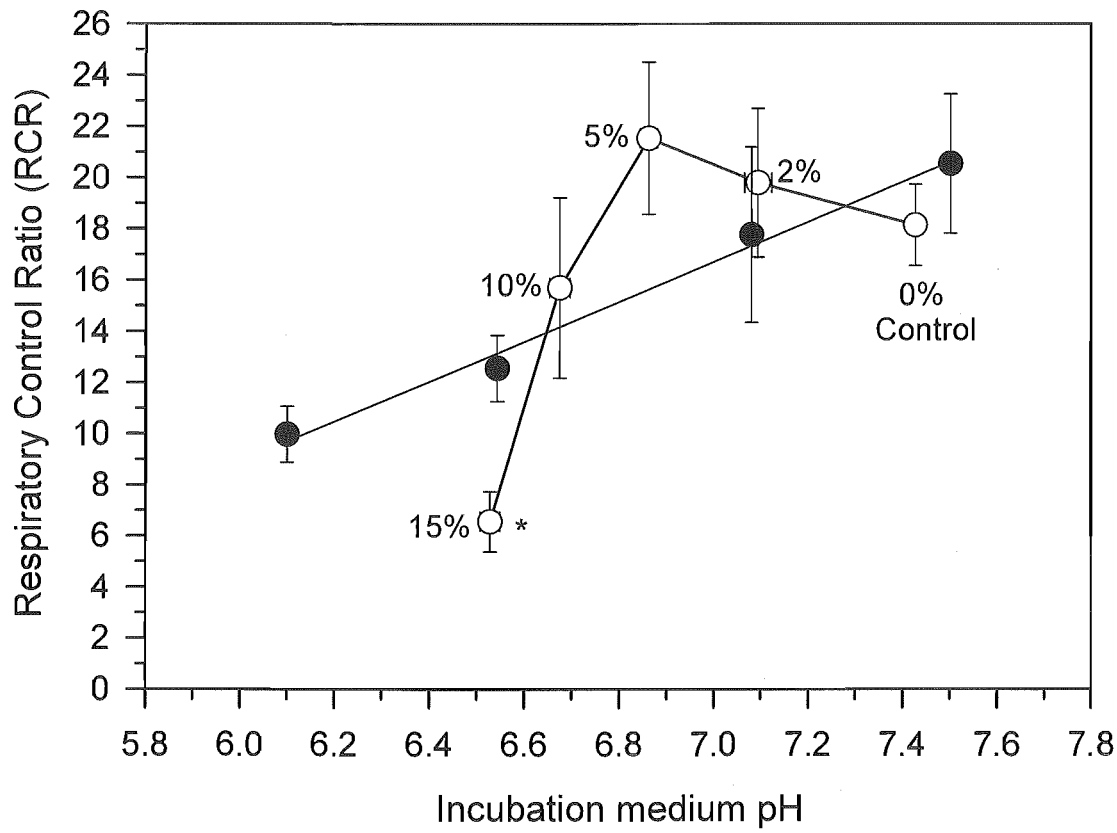

Figure 6.8: *Oxidation of pyruvate by yellow-eye mullet white muscle mitochondria as a function of incubation medium pH alone (●) and incubation medium pH due to various CO_2 concentrations (○). Preparations were incubated at 20.0 ± 0.1 °C. Values are the mean ± SEM, n = 6, unless otherwise stated. * indicates a significantly lower difference from the RCR measured from mitochondria with only a change in incubation medium pH.*

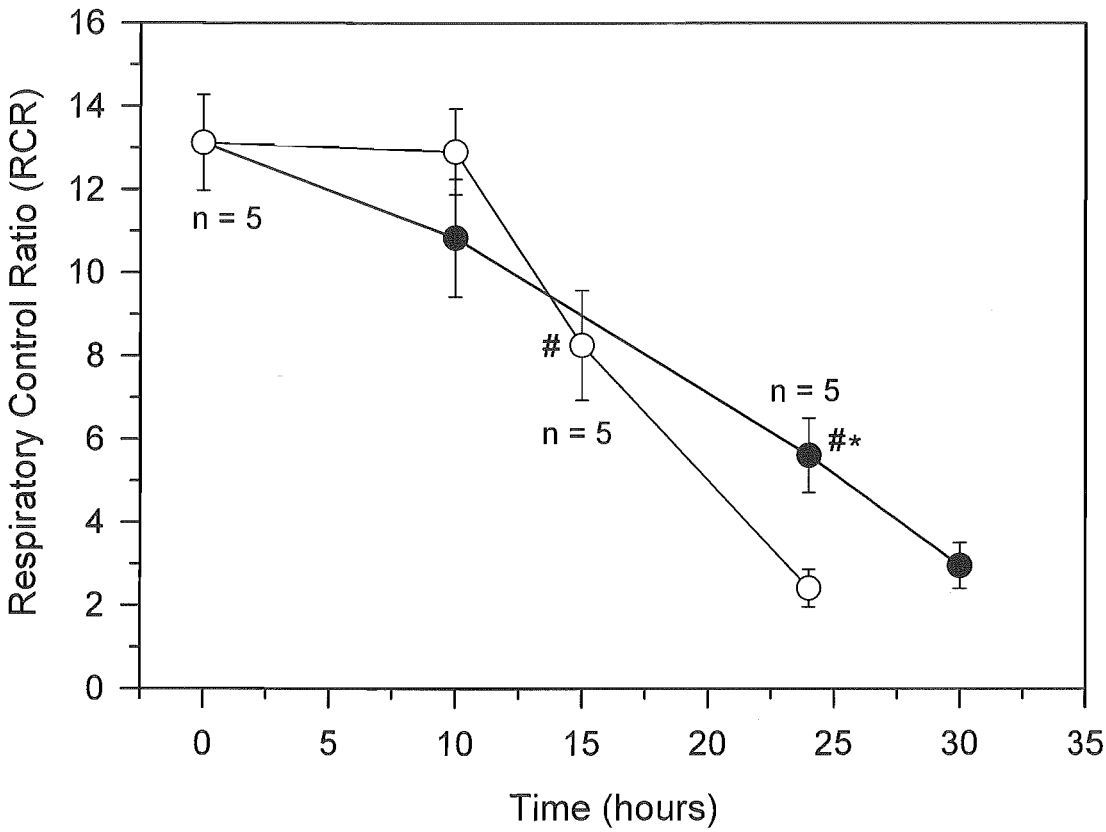

Figure 6.9: *Oxidation of pyruvate by mitochondria extracted from rested post-mortem yellow-eye mullet white muscle stored under normobaric conditions with oxygen flow 50 mL/min ± 1.25% (○) or under hyperbaric conditions 620 ± 10 kPa oxygen flow 50 mL/min ± 1.25% (●). White muscle was stored at half the acclimated temperature (acclimated temperature ranged from 14.0 to 16.2 ± 0.1°C). Mitochondria preparations were also incubated at half the acclimated temperature. Values are the mean ± SEM; n = 6 unless otherwise stated. * Significantly different from the corresponding normobaric value at the same sampling time (Sign test; $P < 0.05$). # Significantly different from the initial pre-storage value – only first instance marked (Sign test; $P < 0.05$).*

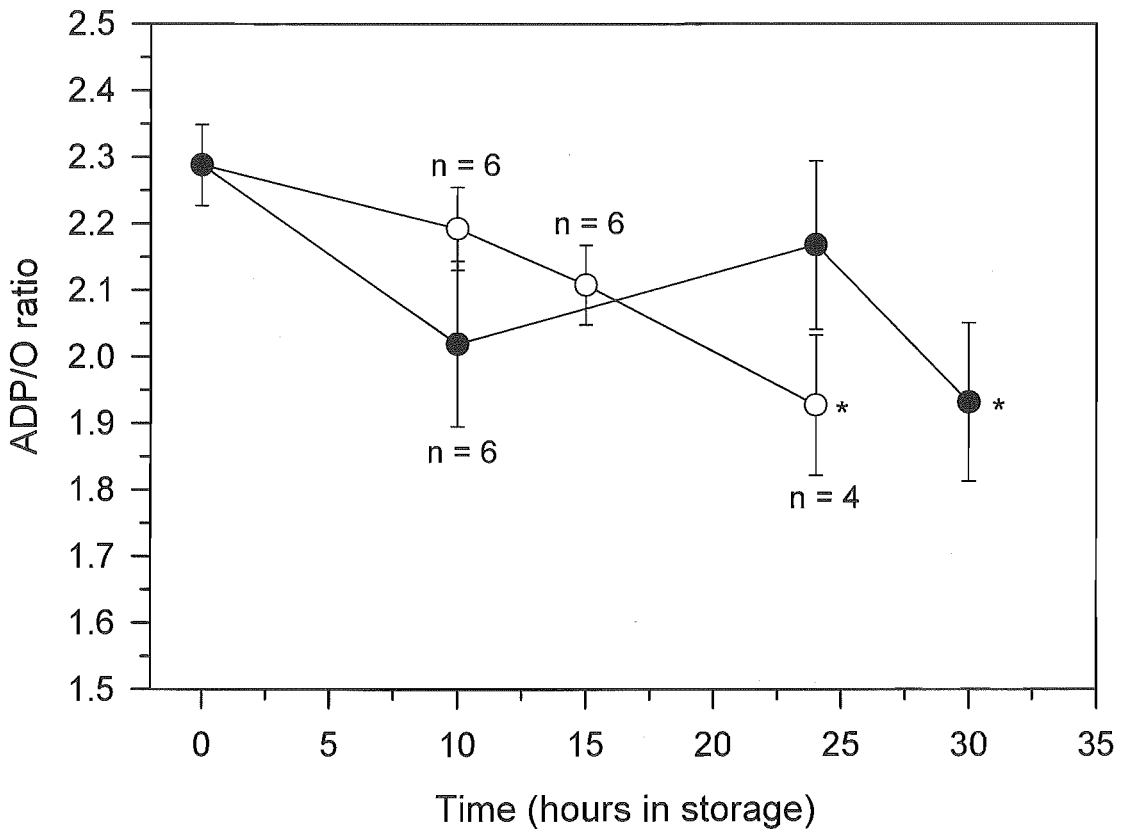

Figure 6.10: *ADP/O ratio during the oxidation of pyruvate by mitochondria extracted from rested post-mortem yellow-eye mullet white muscle stored under normobaric conditions (○) or under hyperbaric conditions (●). For storage details see Fig. 6.9 legend. Values are the mean ± SEM; n = 5 unless otherwise stated. * Significantly different from the initial pre-storage value (P < 0.05; Student's t-test).*

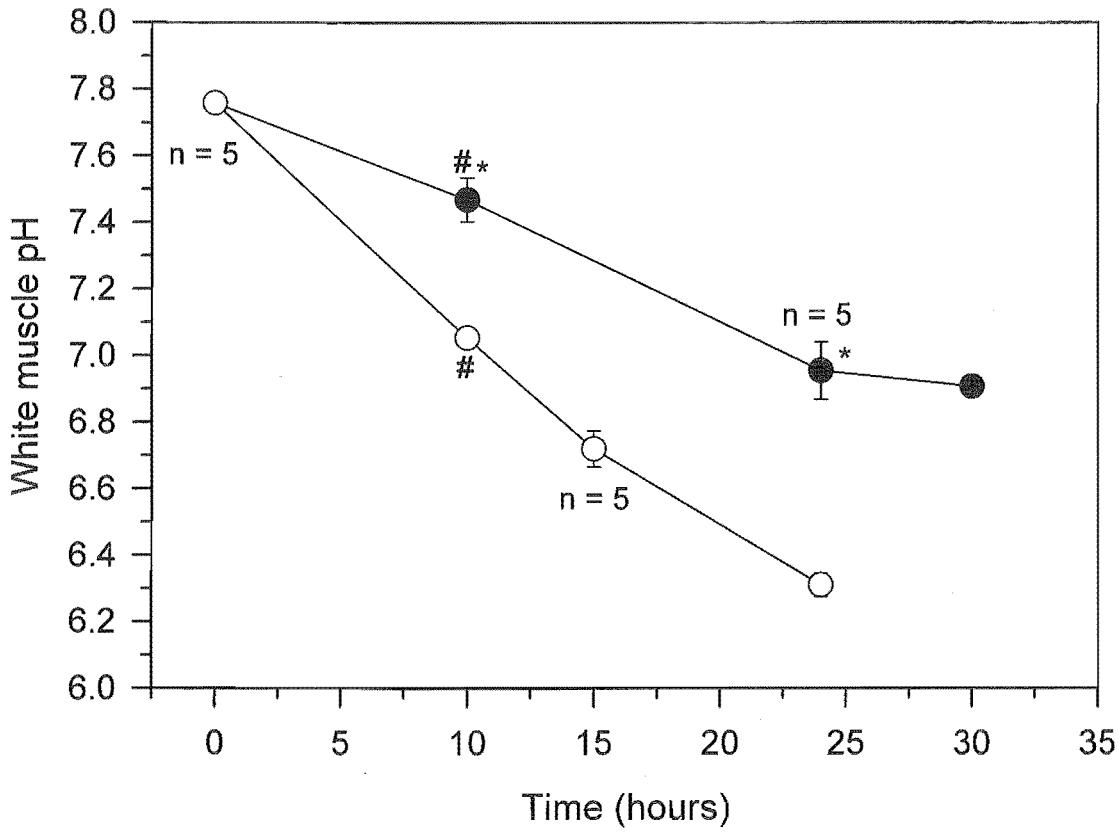

Figure 6.11: *Post-mortem cut-surface pH of the epaxial white muscle from rested yellow-eye mullet white muscle mitochondria stored under normobaric conditions with oxygen flow 50 mL/min ± 1.25% (○) or under hyperbaric conditions 620 ± 10 kPa oxygen flow 50 mL/min ± 1.25% (●). White muscle was stored at half the acclimated temperature (acclimated temperature ranged from 14.0 to 16.2 ± 0.1 °C). Values are the mean ± SEM; n = 6 unless otherwise stated. * Significantly different from the corresponding normobaric value at the same sampling time (Sign test; P < 0.05). # Significantly different from the initial pre-storage value – only first instance marked (Sign test; P<0.05).*

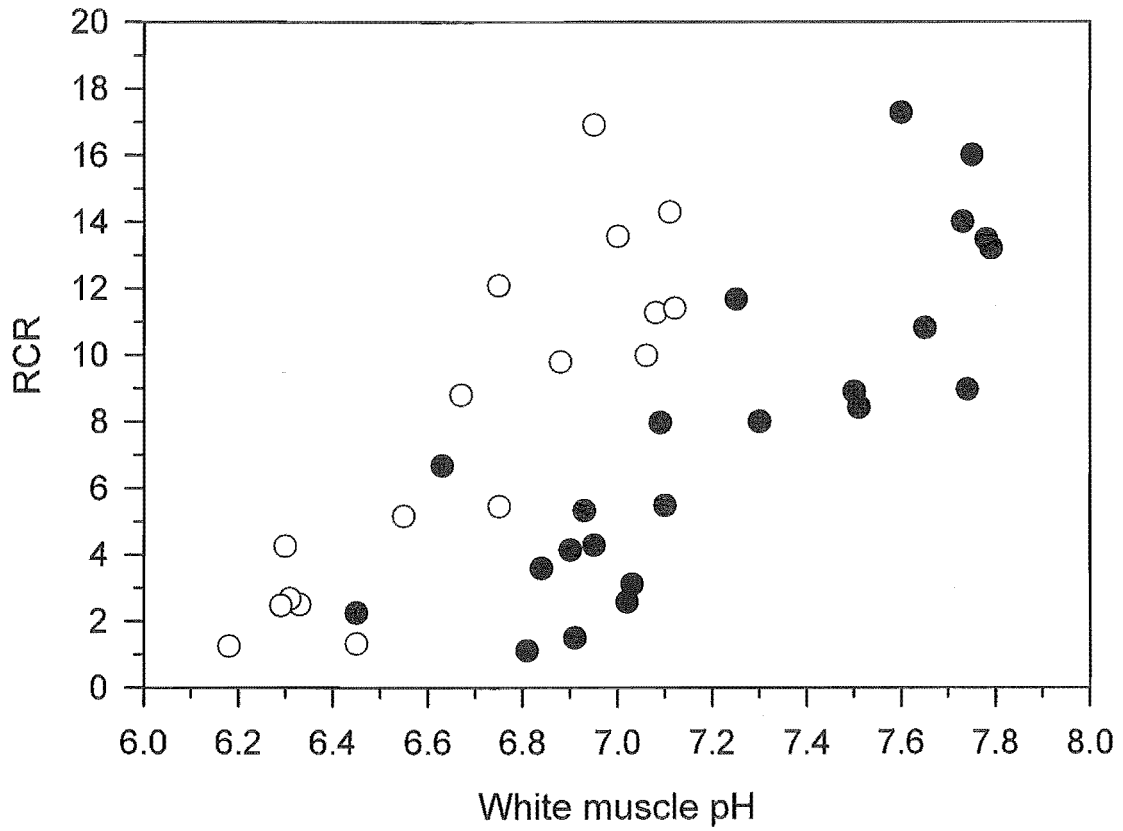

Figure 6.12: *Relationship between respiratory control ratio (RCR) and cut-surface pH from the epaxial postmortem white muscle of the yellow-eye mullet stored under hyperbaric (●) or normobaric conditions (○).*

6.5 DISCUSSION

During hypoxia, inadequate oxygen availability suppresses the rate of mitochondrial ATP production below the energy demands of the tissue. Anaerobic glycolysis is activated with concomitant formation of lactate in order to meet the energy demands of the hypoxic tissue for ATP (Moyes et al. 1995). This was indeed the case in the current ischemic storage experiments when the tissue was stored under normobaric conditions. However, when oxygen availability was not limited (storage under hyperbaric conditions) anaerobic glycolysis was still activated but after a delay. This activation was revealed by the acidification of the WM, accumulation of lactate and depletion of ATP (as shown in Chapter 4, Fig. 4.7).

6.5.1 Methodology

Isolation of mitochondria from the WM of fish is difficult due to the nature of the tissue. The tissue is mainly used anaerobically for burst swimming, and has few mitochondria. This lack of mitochondria meant that a relatively large amount of WM was required to isolate the organelles in numbers similar to those in other studies (Moyes et al. 1989; 1992). The preparation also deteriorated rapidly with time and could only be used for ~1.5 h after the mitochondria had been isolated. The isolation method was not altered to try and improve the "life" of the preparation as the experiments could be carried out in an ~1 h time frame. Although the preparation deteriorated with time the mitochondria were well coupled after extraction (RCR > 10). However, the ADP/O values were lower than the theoretical value of 3 for NAD-linked substrates (Mathews & van Holde 1990) and also lower than those described elsewhere for fish WM mitochondria (2.8 to 3.0, Moyes et al. 1989; 1992). This suggested that although the mitochondria in the current study were well coupled they were probably leaking protons, thus reducing the oxidative phosphorylation efficiency. In both of the Moyes et al. (1989; 1992) studies pyruvate was the main substrate in the mitochondrial preparations, but a "sparker" (malate) was also added to further promote respiration. In the current study the "sparker" was trialed but did not result in any improvement to mitochondrial respiration and was, therefore, abandoned. This may also partially explain the lower ADP/O values

The methods used in this study to measure mitochondrial respiration have been used for many years. However, air saturated incubation medium is very different to what mitochondria would experience in vivo. Oxygen pressures as low as 0.3-0.4 kPa (2-3 mmHg) have been measured in mammalian red muscle under normoxia, thus air saturation results in hyperoxia (rarely physiological) and increases oxidative stress (Gnaiger et al. 2000). The study by Gnaiger et al. (2000) using oxygen-injection microcalorimetry showed that oxidative phosphorylation was more efficient at low oxygen partial pressures than at air saturation. It was not possible to use this new technology in the present study, however, it is believed that the results obtained are useful in describing possible reasons why the WM does not continue to respire aerobically throughout ischemic storage when oxygen was available.

Unfortunately it was not possible to measure mitochondrial respiration under the hyperbaric conditions used for the PM storage experiment due to the limitations of the equipment used. However, as the focus of the study was on the modification of the PM metabolism in the WM fillet a more realistic approach was to store the WM under hyperbaric conditions and then measure mitochondrial respiration at normobaric pressure.

Isoeugenol (the active component of AQUI-S^{TM} and AQUI-S^{TM} Plus) is known to uncouple oxidative phosphorylation from electron transfer in rat liver mitochondria at concentrations greater than 0.44 mM (Cotmore et al. 1979). Results from a study investigating the accumulation of isoeugenol in the muscle of chinook salmon (O. tshawytscha) showed that after 60 min exposure to AQUI-S^{TM} at concentrations of either 17 mg/L or 34 mg/L, residue accumulation was 0.139 µmol/g and 0.097 µmol/g, respectively (Jerrett et al. 1999). Isoeugenol is lipid soluble and can affect membrane permeability (Cotmore et al. 1979), however, it was unclear whether such low concentrations of isoeugenol in the WM would have a detectable effect on the WM mitochondria. It would, therefore, be of interest to extract mitochondria from the WM of fish that have not been exposed to anaesthetic to evaluate any differences in mitochondrial respiration.

6.5.2 Effect of pH on in vitro mitochondria respiration

Intracellular acidosis occurs during sustained exercise and also in the ischemic preparation when the mitochondrial oxygen consumption and free [ADP] are elevated. Prior to starting the pH experiments it was hypothesised that the mitochondria may cease to function at low pH's, (<7.0, 6.5 and 6.1) similar to those experienced during PM storage. This was based on the observation that the ischemic WM preparation apparently switched from aerobic to anaerobic generation of ATP when the cut-surface pH was ~7.1 (see Chapter 4). This, however, was not the case. The RCR decreased as the incubation medium (pH_e) decreased suggesting that the mitochondria were not as well coupled, but they were not inhibited. V_{max} also decreased directly with the decrease in pH_e (Fig. 6.4). However, due to the level of error in the calculations there was only a significant difference in V_{max} at pH_e 6.1 when compared with pH_e 7.5. It was also thought that at the lower pHs the efficiency at which the mitochondria phosphorylated ADP to ATP would drop but this was not the case. There was no significant change in ADP/O ratio at the different incubation medium pHs (Table 6.1). Even though the decrease in RCR with decreasing pH suggested some uncoupling of the mitochondria the amount of oxygen needed to phosphorylate ADP remained the same.

It was difficult to make comparisons between the findings in the present study and other in vitro studies investigating the effects of incubation medium pH on the functioning of mitochondria. Most studies are not carried out on fish, instead, mitochondrial extracts are mainly from mammalian muscle and liver and some from amphibian muscle with very mixed results. The results from a study by Duerr & Hillman (1993) on mitochondria isolated from amphibian skeletal muscle support the current findings in that there was a decrease in V_{max} at pHs below 7.3 and a decrease in RCR below pH 6.9. This pattern also occurred in studies on marine clam mitochondria (Ballantyne & Storey 1983), canine myocardial mitochondria (Mukherjee et al. 1979), and pig muscle and liver mitochondria (Mitchelson & Hird 1973). The current results were, however, in conflict with Moyes et al. (1988) in which the state III and RCR values for carp red muscle mitochondria increased as the pH_e decreased. As discussed by Duerr & Hillman (1993), the result found by Moyes et al. (1988) was not consistent with a chemiosmotic model of oxidative phosphorylation in mitochondria.

A study by Ono et al. (1996) observed that mitochondrial pH rose with external pH and thus matrix pH changed in a parallel fashion with changes in external pH. In the current study when mitochondria were incubated at pHs of 6.1 and 6.5, after a period of State III respiration the incubation medium pH had risen (Fig. 6.4). At the two higher pHs (7.1 and 7.5) there was little change in the pH of the medium. Assuming that the matrix pH changed with the incubation medium, as suggested by the findings of Ono et al. (1996), H^+ could be taken up from the medium into the matrix of the mitochondria, thus increasing the pH. During respiration pyruvate crosses the inner mitochondrial membrane via a carrier that co-transports it with H^+ or exchanges it with OH^- (Mathews & van Holde 1990). Thus during a period of State III respiration H^+ used to co-transport pyruvate would be replaced by those in the incubation medium, further increasing the pH. The higher the $[H^+]$ of the incubation medium the more H^+ would be taken up into the matrix resulting in the large increase in pH after respiration at pH 6.1 and less of an increase at the higher pH (Fig. 6.5). It was assumed that the same amount of H^+/OH^- ions would be required to transport pyruvate across the inner mitochondrial membrane during a period of State III respiration at all incubation medium pHs, with the differences in pH changes being due to the equilibration of the matrix pH to the incubation medium pH.

In a study carried out by Tonkonogi and Sahlin (1999) it was observed that lactic acidosis had different effects on rat skeletal muscle mitochondria when induced on nonphosphorylating versus actively phosphorylating mitochondria. When the incubation medium was acidified prior to the addition of ADP (nonphosphorylating) the maximal rate of oxidation was lowered. However, if the acidification occurred during active phosphorylation (state III) there was no change to V_{max}. When the incubation medium was acidified prior to phosphorylation there was also an increase in sensitivity to ADP. In the present study the incubation medium was acidified prior to phosphorylation and therefore it is possible that the mitochondria may not have been respiring at their maximal rate. Unlike the Tonkonogi & Sahlin findings, the mitochondria isolated from yellow-eye mullet WM did not have an increased state IV oxygen consumption. The decrease in RCR with pH was due more to the decrease in V_{max}.

From the results in the current study it appeared that an increase in hydrogen ion concentration (low pH) did not prevent isolated mitochondria from respiring in State III but low pH did depress the rate of oxidation. Therefore, it was likely that a drop in pH alone would not explain the increasing lactacidosis observed in the PM hyperbaric treated WM. These results must be tempered with an understanding of the limitations of the in vitro method as mitochondria in vivo never respire at the State III rate.

6.5.3 Effect of CO_2 on in vitro mitochondria respiration

Because the pH of the incubation media changed with CO_2 concentration (pH decreased as CO_2 increased) the true effect of CO_2 alone on mitochondrial respiration was not measured. However, the results from the current study provide some interesting discussion points. When mitochondria were exposed to various levels of CO_2 in the incubation medium, RCR and V_{max} only became depressed at the highest concentration of CO_2 (112.5 torr/15%, Figs. 6.5 & 6.6). This concentration of CO_2 was unlikely to be a realistic level experienced by the cells in vivo. In a study carried out by Wang et al. (1998) using a perfused trout trunk preparation, WM intracellular CO_2 partial pressure (P_{ICO_2}) was calculated at rest and after exercise (electrical stimulation). At rest P_{ICO_2} was ~7.5 torr rising to ~12.5 torr 5 min after exercise. The level of P_{ICO_2} reached even after exercise was not quite 2% CO_2 (15 torr). The results obtained from the present in vitro study would suggest that CO_2 on its own would not inhibit oxidative phosphorylation at physiological levels.

Although an in vivo concentration of 15% CO_2 in the current study was unrealistic, the mitochondria were respiring under other in vitro experimental conditions that were also unrealistic. Mitochondria were incubated in medium with unrealistically high concentrations of external O_2 and CO_2 and also unrealistic levels of substrate (pyruvate). It is possible that the mitochondrial drive to respire under these conditions was also much greater than that experienced in vivo, remembering that mitochondria do not respire at the state III rate in vivo. To inhibit mitochondrial respiration under these unrealistic conditions, may therefore, require unrealistic levels of substances that may normally inhibit mitochondria in vivo at much lower (physiological) levels.

Significance of pH ~6.8

In light of this, there appeared to be a distinct inflection point in the RCRs of mitochondria incubated in medium equilibrated to between 5 and 10% CO_2, and a significant drop at 15% (Fig. 6.6 & 6.8). This inflection point corresponded with an incubation medium pH of ~6.8. Coincidentally, when fish are forced to swim to exhaustion, for example during harvesting, the immediate pH of the WM is very rarely lower than 6.8 (Jerrett et al. unpublished results for yellow-eye mullet, chinook salmon and snapper). It is thought that if the WM pH does drop below 6.8 then the chance of the fish recovering from the exercise bout is minimal. Because recovery of the WM relies on aerobic generation of ATP it is not unreasonable to suggest that below pH 6.8 the mitochondria may be affected in such a way that prevents oxidative phosphorylation.

In mitochondria, as a result of Ca^{2+} accumulation, there can be sudden permeability increase of the inner membrane called the permeability transition (PT). The PT is mediated by the opening of a specific, non-selective high-conductance inner membrane channel, the membrane transition pore (MPT). Above pH 7.0 in normally functioning mitochondria the pores of the inner membrane may constantly "flicker" open and closed in order to maintain ion gradients for optimum operating efficiency. However, the PT is potently inhibited at matrix pH below ~7.0 (Bernardi 1999). Although the pH of the WM can drop below 7.0 when fish are swum to exhaustion (presumably preventing the PT) the blood pH does not. During recovery the WM is re-perfused with extracellular fluid above pH 7.0. Along with increased $[Ca^{2+}]$ in cells during exercise, the PT is also promoted by pH>7.0. It is possible that if the WM pH was too acidic (i.e. <6.8) on re-perfusion that an irreversible PT would occur due to the alkaline re-perfusing plasma, resulting in cell death and eventually be lethal to the whole animal. Hence the limit on how low the pH of the WM can be safely driven during exercise. It may not be surprising then that a pH of ~6.8 is significant for the mitochondria.

Occurrence of free CO_2 in the white muscle

In chapter 3, restricting oxygen flow through the hyperbaric treatment chamber essentially counteracted the delay in acidification possible with hyperbaric hyperoxia

and gas flushing. As discussed in the Introduction, this result suggested that elimination of waste products was an issue for the continuation of aerobic generation of ATP, lending weight to inhibition of mitochondrial respiration by CO_2 and other waste products of aerobic respiration. In resting trout muscle elimination of CO_2 out of the cell is mediated by cytoplasmic carbonic anhydrase (CA; Wang et al. 1998). CA maintains an instantaneous equilibrium between CO_2 and HCO_3^-. As CO_2 diffuses across the sarcolemma out of the cell, it is immediately replaced from the much larger but less permeable intracellular HCO_3^- pool (Wang et al. 1998). To maintain the trans-membrane P_{CO_2} gradient, CA on the extracellular surface catalyses the hydration of CO_2 to HCO_3^-. In the current in vitro preparation the high level of CO_2 of the incubation medium (acting as the cytoplasm) would prevent adequate diffusion of CO_2 out of the mitochondria, possibly leading to an inhibition of oxidative phosphorylation due to a build up of waste product. Although CA is also compartmentalised in the mitochondria (Wang et al. 1998) and would be able to hydrate CO_2 to HCO_3^-, the combination of high $[H^+]$ and high $[CO_2]$ in the incubation medium would drive dehydration of HCO_3^- back to CO_2, again increasing the $[CO_2]$ of the matrix and possibly disrupting oxidative phosphorylation.

Overall, as the WM cells became acidified under hyperbaric, hyperoxic storage conditions any CO_2 produced during aerobic generation of ATP would have begun to dominate as free CO_2 rather than HCO_3^-. As the $[CO_2]$ increased with the increasing $[H^+]$ there may have been an inflection point below pH 7.0 where cellular conditions are such that oxidative phosphorylation is inhibited. This may be due to transport of CO_2 out of the mitochondria and out of the cell being limited because of disrupted trans-membrane gradients, or, as in Chapter 3, by inadequate gas flow to allow the CO_2 to diffuse out of the tissue as it is being generated. Beyond pH ~6.8 mitochondria may not just be inhibited, they may be damaged, as suggested by the poor coupling of mitochondria incubated in medium equilibrated with 15% CO_2 and subsequent pH of 6.52 (Fig. 6.8).

6.5.4 Post-mortem changes in mitochondria respiration

Measurement of the RCR of mitochondria extracted from the WM at various stages during PM storage showed that even when WM is stored under normobaric conditions the mitochondria do not begin to deteriorate (become poorly coupled) until after 10 to

15 h storage (Fig. 6.9). This was somewhat surprising due to the fact that ATP levels in the WM held under normobaric conditions start to be depleted immediately upon PM storage (Chapter 4: Fig. 4.7b). ATP levels dropped to around half the pre-storage level after 10 h storage under normobaric conditions. It was assumed that the mitochondria would become progressively less-coupled through PM storage due to depletion of ATP and acidification of the WM. In comparison, WM stored under hyperbaric conditions still had high levels of WM ATP after 10 h storage (Chapter 4: Fig. 4.7b) and showed very little acidification (Chapter 4: Figs 4.5 and 4.6). Thus, it would be expected that the WM mitochondria would be well-coupled at this stage due to the minimal disturbance to the WM.

In WM held under normobaric conditions, the RCR of the mitochondria after 15 h storage had significantly dropped to below the pre-storage level. However, with an RCR of 8.3 ± 1.3, this was still considered to be well-coupled. After 24 h storage the mitochondria were considered to be poorly coupled (Fig. 6.9). Storage of WM under hyperbaric conditions resulted in the mitochondria RCR dropping below pre-storage levels after 24 h and they were poorly coupled after 30 h. The ADP/O ratio, (that is the amount of oxygen required to phosphorylate the ADP added to the mitochondrial suspension) decreased steadily during PM storage in normobaric WM, showing a positive correlation with RCR. However, under hyperbaric storage conditions there was no significant change in ADP/O ratio from the pre-storage level until after 30 h storage. The variation in the ADP/O measurement made it difficult to draw conclusions from the data, but it would seem that PM storage under hyperbaric conditions may allow phosphorylation of ADP with lower levels of oxygen for longer (i.e. higher ADP/O ratio). As discussed in Chapter 4 Section 4.5.5, yellow freshwater eels acclimated to high hydrostatic pressure (10.1 MPa) can increase the efficiency of oxidative phosphorylation (increased ADP/O; Theron, et al. 2000). In the current study, hyperbaric PM storage was used to deliver substrate to the WM rather than to induce changes in energy metabolism as occurs with high hydrostatic pressure acclimation. As the ADP/O ratio in hyperbaric WM did not increase during PM storage it would suggest that oxidative phosphorylation did not increase in efficiency, rather it was able to continue for longer compared with normobaric storage of the WM. It was also difficult to draw conclusions from the data of the hyperbaric treatment in this experiment as the

mitochondrial respiration measurements were not made under the same pressure as the WM storage pressure.

Although the difference in cut-surface WM pH was quite large during PM storage under normobaric and hyperbaric conditions (Fig. 6.11), the differences between RCRs was not as great (Fig. 6.9). This suggested that although the WM stored under hyperbaric conditions was able to use oxygen for oxidative generation of ATP (ATP levels were maintained for 25 h PM storage Chapter 4: Fig. 4.7b) the conditions were not wholly protective of the mitochondria. The RCR had significantly dropped after 24 h storage to 5.61 ± 0.89, despite the WM pH of 6.95 ± 0.09. In WM stored under normobaric conditions the RCRs were still 8.3 ± 1.3 after 15 h storage when the WM pH was 6.74 ± 0.05. Throughout the PM storage trial, RCRs for mitochondria extracted from WM stored under normobaric conditions were higher at lower cut-surface pH's than mitochondria from WM held under hyperbaric conditions (Fig. 6.12).

6.5.5 Significance in the live animal

During burst swimming in which the WM becomes hypoxic, the mitochondria are not in use due to a lack of substrate, i.e. oxygen. The mitochondria are, in a sense, 'on hold' in that situation, waiting for the blood supply to return and the oxygen with it. The mitochondria are able to resume their function once conditions are favourable, undamaged and well-coupled. Storage of WM under hyperbaric conditions with very high levels of oxygen is a non-physiological scenario that fish muscle would never experience. As outlined at the start of the discussion, oxidative phosphorylation is actually more efficient at low oxygen levels. Under very high oxygen levels, it seems that the mitochondria may be 'forced' to function and produce ATP oxidatively.

6.5.6 Potential problems with high levels of oxygen

Excess oxygen in the cell can create problems despite apparently sufficient aerobic ATP production rates. As mentioned in Chapter 4, high levels of oxygen in tissue can be hazardous to the life of the cell, due to oxygen's excellent electron-accepting properties with the formation of reactive oxygen species i.e. $O_2\cdot^-$, H_2O_2, $OH\cdot$ (Jamieson et al. 1986; Skulachev 1996). The cell has several levels of anti-oxygen defence including those that inactivate reactive oxygen species (ROS). These are superoxide dismutase,

catalase, peroxidase and antioxidants (e.g. ascorbic acid, tocopherol etc). Isoeugenol, the active component of AQUI-S^{TM}, also has significant hydroxyl radical scavenging activity (Rajakumar & Rao 1993). However, actual reduction of the O_2 concentration may be a more appropriate way of reducing the toxic effects of too much O_2. An hypothesised theory for reduction of $[O_2]$ in the tissue is by "mild-uncoupling" of the mitochondria (Skulachev 1995, 1996) resulting in H^+ leakage across the mitochondrial membrane. The mitochondrial F_1F_0-ATPase acts as the site for ATP production. This reversible enzyme, the ATP synthase, can run backwards and actively pumps protons from the matrix in an attempt to maintain the mitochondrial membrane potential (Boutilier 2001). It has been demonstrated that a small decrease in the mitochondria membrane potential, due to mild-uncoupling, switches off $O_2^{\cdot-}$ accumulation in the mitochondria (Papa & Skulachev 1997). Overall, mild-uncoupling would prevent complete inhibition of respiration and accumulation of the one-electron O_2 reductants during State IV (after all ADP had been phosphorylated), along with decreasing the intracellular $[O_2]$. If this defence system is insufficient there is a more powerful system to remove oxygen. It has been suggested by Papa & Skulachev (1997) this maybe performed by non-specific pores in the inner membrane of the mitochondria (previously discussed in Section 6.5.3). These pores are cyclosporin A-sensitive and are permeable to compounds of molecular mass up to 1500 Da. Opening of the non-specific pore occurs under conditions of mitochondrial calcium overload, and is exacerbated by oxidative stress, adenine nucleotide depletion and decreased membrane potential (Halestrap et al. 1997). This is known as the mitochondrial permeability transition (MPT). The pores convert the mitochondria from being "power stations" to an O_2 and substrate consuming furnace without any energy storage (St-Pierre et al. 2000). Mitochondria with open pores are also able to hydrolyse the ATP made by glycolysis and the remaining functional mitochondria.

In the current study, the assumed high concentration of O_2 in the WM stored under hyperbaric conditions may have resulted in ROS formation in the mitochondria, the consequences of which may have been mild-uncoupling to reduce the O_2 concentration. This maybe the cause of the reduced RCRs in mitochondria from hyperbaric WM at pHs while mitochondria from normobaric WM were well-coupled. Although supplying oxygen to the WM allows for an extended period of ATP

production via oxidative phosphorylation, it may result in premature uncoupling of the mitochondria.

The experiment in the current study can be characterised as "stress on stress" (SOS) studies (Viarengo et al. 1995) with the stress associated with acute exposure to high oxygen pressure overlaid on PM anoxia and ischemia. These parameters were, in turn applied to rested muscle tissue to avoid substantial artefacts associated with muscle fatigue (Jerrett et al. 1996; 2000). Previously, the hyperbaric, hyperoxic conditions were not viewed as a stress as it resulted in a benefit to the WM during PM storage. However, as it appears that the mitochondria maybe prematurely damaged/uncoupled because of the high pressure treatment, it could be viewed as a stress.

From the results of this chapter and previous chapters, the most likely explanation for the change from aerobic to anaerobic generation of ATP in WM stored under hyperbaric, hyperoxic conditions is that the mitochondria may become damaged due to the high levels of oxygen, in turn inhibiting oxidative phosphorylation. At the same time, aerobic generation of ATP would result in generation of waste products, such as CO_2 and H^+. The combination of these waste products possibly disrupt trans-membrane gradients and equilibriums in the mitochondria leading to further inhibition of oxidative phosphorylation.

6.5.7 Questions to arise from these experiments

The results from the current study give an indication that acidification of the WM alone may not inhibit aerobic generation of ATP. However, in combination with CO_2, a waste product of oxidative phosphorylation, there appear to be conditions that inhibit mitochondrial respiration that coincide with a pH of ~6.8, a pH that corresponded with WM pH limits during exercise, and possible MPT events in the mitochondria. Normobaric conditions appear to protect the functionality of the mitochondria more so than hyperbaric storage. Under hyperbaric conditions it was possible that the high levels of oxygen in the tissue actually "poisoned" the mitochondria as well as forcing them to respire. The question then arises as to whether there is a more appropriate level of oxygen that could be delivered to the WM (e.g. 0.3-0.4 kPa as described by Gnaiger et al. 2000) that would limit the amount of free radicals produced and the rate of CO_2

and H^+ production but still allow normal respiration to continue. It is certainly an area of research that demands attention.

6.6 SUMMARY

When mitochondria were extracted from the WM and exposed to incubation medium pHs similar to those experienced by the WM during PM storage, there was only a minimal amount of respiratory inhibition at pH 6.5. Similarly, when exposed to various levels of CO_2 in the incubation medium (1 to 10%) there was no disturbance to in vitro respiration. Significant inhibition only occurred after exposure to 15% CO_2. However, the high in vitro levels of CO_2 and low pH did inhibit respiration to a greater degree than did low pH alone. While not statistically significant, the relationship of CO_2 and medium pH to inhibition did not appear to be linear with increased inhibition occurring below pH ~7 to 6.8 and 10 to 15% CO_2. This is coincident with the pH WM will commonly attain after forced, exhaustive exercise and a pH that may result in a PT during reperfusion on recovery and requires further investigation.

When the WM was stored under normobaric conditions the mitochondria became uncoupled after 24 h. It was expected that the mitochondria from the WM stored under hyperbaric conditions would remain well-coupled for much longer than under normobaric conditions. However, there was only a 6 h extension before mitochondria were uncoupled (30 h). This may be attributed to the observation that under hyperbaric storage conditions there is a period of aerobic metabolism in which the mitochondria would generating ATP.

From the results of this chapter and previous chapters, the most likely explanation for the change from aerobic to anaerobic generation of ATP in WM stored under hyperbaric, hyperoxic conditions is that the mitochondria may become damaged due to the high levels of oxygen, inhibiting oxidative phosphorylation. At the same time, aerobic generation of ATP would result in waste product generation of CO_2 and H^+. The combination of these waste products possibly disrupted trans-membrane gradients and equilibriums in the mitochondria leading to further inhibition of oxidative phosphorylation.

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CHAPTER 7

The effect of temperature acclimation on the post-mortem metabolism of white muscle stored under hyperbaric and normobaric conditions.

7.1 ABSTRACT

Fish undergo a series of compensatory biochemical modifications during cold acclimation. The compensation usually occurs over the course of several weeks during which the maintenance of aerobic flux is paramount. In Chapter 4 it was noted that PM storage of WM under hyperbaric oxygen conditions resulted in a slowing of PM metabolic acidosis due to an initial period of aerobic metabolism. It was hypothesised that cold acclimated fish would have an increased period of aerobic metabolism with cold acclimation when stored under hyperbaric conditions due to their (reported) biochemical modifications to maintain aerobic flux. The effect of acclimation temperature on the PM changes was studied in yellow-eye mullet acclimated to natural summer (20.1 ± 0.1 °C) and winter (12.1 ± 0.1 °C) temperatures. Fillets were stored at 620 ± 10 kPa or at atmospheric pressure. Both treatments were supplied with humidified oxygen flow of 50 mL/min ± 1.25%. Cut-surface pH, lactate, ATP, P_i, creatine and glycogen were measured in the WM throughout the PM storage period. There were no differences in these parameters in the summer and winter acclimated WM held under normobaric conditions. Winter acclimated WM did not have an extended aerobic period under hyperbaric conditions as predicted. PM changes in this treatment were similar to those in normobaric summer WM. Winter acclimated WM had lower levels of creatine than the summer acclimated fish. The results suggested that the winter acclimated WM relied primarily on carbohydrate for ATP generation compared with a possible mixture of both carbohydrate and lipid in summer acclimated WM during storage under hyperbaric conditions. Summer acclimated WM had a greater aerobic scope than winter acclimated WM. One possible reason may be that summer acclimated WM has access to greater reserves of both carbohydrate and lipid due to seasonal metabolic machinery differences. Behavioural changes associated with

cold acclimation in the mullet and further evidence from species that are relatively inactive in winter are discussed in relation to these findings.

7.2 INTRODUCTION

In Chapter 3 several questions arose as to the effect of acclimation temperature on the PM metabolism of the WM. Rested winter acclimated mullet had higher blood and WM pH values, but, the rate of WM acidification during normobaric storage was faster. Differences between summer and winter acclimated mullet were apparent in blood pH measurements and also in the PM WM pH measurements. The question then arose: if the temperature can affect the pH of the blood and the WM then how does this relate to PM metabolism? The objective of the current chapter was to address this question and determine if acclimation temperature could modify PM metabolism in the WM.

During the course of a year fish can face large fluctuations in water temperature and other environmental conditions in their natural habitat. Mature fish also have to change their metabolic focus by directing energy intake into somatic muscle growth for a certain period and then channel it into gonad development prior to spawning. To be able to do this requires significant changes in the metabolic machinery of fish usually through alterations in enzyme activities and levels in various organs and muscles. For example, in rainbow trout, transformation from the non-spawning to spawning phase was associated with a dramatic change in the activity of enzymes studied in the WM (Kiessling et al. 1995). Glycolytic capacity decreased to less than half during spawning while oxidative metabolism increased two- to four-fold.

Although nutritional status can considerably alter the metabolism of fish, so can changes of temperature. It has been well documented during cold acclimation that over the course of several weeks fish undergo a series of compensatory biochemical modifications (for a review, see Johnston 1993). As the physio-chemical properties of cells and body fluids change with temperature, the fish has various mechanisms to counter these physical changes in order to remain active at cold temperatures.

Historically it was often assumed that poikilotherms operate at lower metabolic rates in colder habitats and seasons. However, in nature many cold-blooded animals function relatively independent of temperature over relatively broad temperature ranges (Bullock 1955). Some studies have produced conflicting results for the same species regarding their compensatory changes on cold acclimation (Table 7.1). The majority of studies found that the levels and activities of some glycolytic enzymes increased with cold

acclimation and/or there were increases in mitochondrial volume density in red and white muscle (Battersby & Moyes 1998; Egginton & Sidell 1989; Guderley & Johnston 1996; Hubley et al. 1997; St-Pierre et al. 1998). A summary of changes to morphology, enzymes and mitochondrial density is shown in Table 7.1.

Many of the studies mentioned were carried out on species that are naturally anoxia tolerant (i.e. carp and goldfish) and/or active at low temperatures (salmonids, carp and goldfish). Some anoxia tolerant species have developed alternate anaerobic biochemical pathways that have less toxic end-products than conventional pathways, such as the production of ethanol instead of lactate (Shoubridge & Hochachka 1980; van den Thillart & van Raaij 1995). Therefore, it is not surprising that these species make changes to their physiology to remain active at low temperatures as well.

To be active at low temperatures it is necessary to conserve the capacity for aerobic metabolic flux. The changes occurring at the mitochondrial level (increased density and enzyme activity) suggest that aerobic generation of ATP becomes limiting at low temperatures and therefore the muscle has to compensate for the reduced catalytic rates of mitochondrial enzymes (Johnston 1982; Egginton & Sidell 1989). By increasing the mitochondrial density the mean diffusion path lengths between mitochondria and cytosolic compartments are reduced, compensating for the reduced diffusion coefficients of cytosolic metabolites at low temperatures (Johnston 1982; Egginton & Sidell 1989; Hubley et al. 1997). Increasing the activity of some enzymes in the glycolytic pathway ensures that the flux through the pathway is maintained even though some enzyme reaction rates may be affected by temperature, e.g. phosphofructokinase (Lehoux & Guderley 1997).

These changes allow the metabolic rate of cold-acclimated animals to be similar to warm-acclimated animals of the same species instead of being slower at colder temperatures. Thus, the animal compensates for chemical reactions occurring more slowly at lower temperatures allowing the animal to remain active. In contrast, some species such as rainbow trout do not increase their aerobic capacity on cold acclimation. This suggests that they have a sufficiently high muscle aerobic capacity to fulfil the requirements of sustained swimming at low temperatures (Thibault et al. 1997, Table 7.1).

Several studies have looked at the relationship between acclimation temperature and the progression of rigor mortis (Abe & Okuma, 1991; Hwang et al. 1991; Watabe et al. 1990). These studies found that the rate of rigor mortis was dependent on the difference between acclimation and storage temperature. In a study by Lee et al. (1998), using cultured red sea bream and Japanese flounder acclimated to 25 °C, it was shown that progression of rigor mortis was slowed with a decrease in storage temperature from 25 °C to 12 °C (half the acclimated temperature). However, when stored below 10 °C progression of rigor mortis became faster. Simple thermodynamic principles would suggest that the colder the storage temperature, the slower the reaction rate should be.

In the author's laboratory it has been identified that different species of fish acclimated to different temperatures have different optimum PM WM storage temperatures (chinook salmon: Oncorhynchus tshawytscha, Jerrett et al. 2000; yellow-eye mullet: Aldrichetta forsteri, Law & Jerrett 1996; snapper: Pagrus auratus, Jerrett, et al. 2002, see also Chapter 3 Materials and Methods). Jerrett et al. (2000) found similar results to Lee et al. (1998) in chinook salmon naturally acclimated to summer (18.8 °C) and winter (10.7 °C) temperatures. In general, it appeared that storage at half-the acclimated temperature of the fish was the optimum temperature for retarding ATP depletion during the PM storage of rested chinook salmon, mullet and snapper WM. In the case of chinook salmon there was no increase in PM metabolic rate below half the acclimated temperature until the muscle began to freeze. In snapper and mullet WM the PM metabolic rate did increase below half the acclimated temperature. Jerrett et al. (2000) also found that winter acclimated chinook salmon were 2.2 times more sensitive to any change in PM storage temperature than summer acclimated fish. Winter acclimated mullet were 1.6 times more sensitive and snapper were 1.53 times more sensitive (Jerrett et al. unpublished data). The results from these experiments have had extensive impact on the current work, allowing the PM storage trials to be conducted at temperatures in the optimum range for tissue preservation.

The differences between species in PM metabolic changes at chilled storage temperatures in summer and winter acclimated fish outlined above and the metabolic reorganisation in the muscle reported in the literature, prompted further investigation into the differences in PM metabolism of summer and winter acclimated fish. The

results from Chapter 4 showed that it was probable that the WM stored under hyperbaric conditions was able to respire aerobically in the first 12 to 27 h of PM storage. As the compensatory mechanisms that can occur during cold acclimation are related to the maintenance of aerobic flux, it was of interest to determine if mullet naturally acclimated to winter temperatures received more benefit (i.e. further extension of aerobic metabolism) from PM storage under hyperbaric conditions. Thus, the objective of the current study was to follow the metabolism of PM WM from summer and winter acclimated yellow-eye mullet (A. forsteri) stored under the standard storage protocols (hyperbaric conditions 620 ± 10 kPa with humidified oxygen flow set to 50 mL/min ± 1.25% and under normobaric conditions with humidified oxygen flow set to 50 mL/min ± 1.25%). PM metabolism of the WM was followed by measuring indicators of PM change (cut-surface pH, WM lactate, P_i, ATP, creatine and glycogen). Mitochondria were also extracted from summer and winter acclimated fish to determine if any differences in aerobic metabolism existed at the cellular level.

Table 7.1: *Changes in mitochondria morphology, density and enzyme activity upon cold acclimation in various fish species.*

			Changes on cold acclimation			
Species	**Acclim temp**	**Muscle type**	**Morph change**	**Incr. mitochondria density**	**Enzyme change**	**Reference**
Rainbow trout (Oncorhynchus mykiss)	Natural	white & liver	-	-	None	Thibault et al. (1997)
Rainbow trout (Oncorhynchus mykiss)	4 & 18°C	red & white	-	-	Increased	Battersby & Moyes (1998)
Rainbow trout (Oncorhynchus mykiss)	1 & 16°C	Red	no	-	Increased	St. Pierre et al. (1998)
Rainbow trout (Oncorhynchus mykiss)	4, 11 & 18°C	red & white	no	yes	-	Egginton et al. (2000)
Rainbow trout (Oncorhynchus mykiss)	Natural	Red	-	-	Increased	Guderley & St. Pierre (1999)
Crucian carp (Carassius carassius)	5 & 25°C	Red	yes	yes	-	Kilarski et al. (1996)
Carp (Cyprinus carpio)	10 & 28°C		-	-	None	Wodtke (1976)
Goldfish (Carassius auratus)	5 & 25°C	red & white	-	yes	-	Hubley et al. (1997)
Striped bass (Morone saxatilis)	5 & 25 °C	red & white	no	yes	-	Egginton & Sidell (1989)
Short-horned sculpin (Myoxocephalus scorpius)	5 & 15°C	Red	-	-	Increased	Guderley & Johnston (1996)
Sea bass (Dicentrarchus labrax)	10 & 22°C	liver & heart	-	-	Decreased	Trigari et al. (1992)
Order: Perciformes	Winter & Summer	Red	yes	yes	-	Johnston et al. (1998)
Channel catfish (Ictalurus punctatus)	7, 15 & 25°C	white & liver	-	-	Increased	Seddon (1997)
Threespine stickleback (Gasterosteus aculeatus)	8 & 23°C	Red & white			Increased	Guderley & Leroy (2001)

7.3 MATERIALS AND METHODS

7.3.1 Experimental timing and acclimation temperatures

Fish were reared under the same conditions as described in Chapter 3.
In the experiments to determine the effect of natural acclimation temperature on PM metabolism under hyperbaric conditions mullet were sampled from the same population in September 1998 (winter acclimated; see Discussion: Section 7.5.1) and again in December 1998 (summer acclimated).

The mean daily seawater temperature experienced by the fish in the 14 d prior to the September 1998 sample (winter) was 11.5 ± 0.1 °C (± SEM). The mean seawater temperature for the 14 d prior to the December 1998 sample (summer) was 18.5 ± 0.3 °C (± SEM).

7.3.2 Fish capture and sampling

Fish were anaesthetised with AQUI-S™ Plus at a concentration of 30 ± 0.5 mg/L (see Chapter 2: Harvesting Method). Once fish were anaesthetised a mixed venous blood sample was taken and the pH measured along with lactate and glucose (see Chapter 2: "Blood sampling and pH measurement).

7.3.3 Fillet storage

The fish were pithed immediately after the blood sample was taken. The WM was excised from the carcass and the fillets were stored either under hyperbaric conditions (LHS fillets stored pressurised to 620 ± 10 kPa with oxygen flow set to 50 mL/min ± 1.25%) or normobaric conditions (RHS fillets held at ambient pressure with oxygen flow set to 50 mL/min ± 1.25%) as described in Chapter 3. The WM was stored at half the ambient temperature on the day of experimentation as this has been shown to slow PM changes without placing hypothermic stress on the tissue and allows resolution of the changes (see Chapter 3 Section 3.6: "Choice of storage temperature"). The fish used in the current study were the same as those used in Chapter 3: rested winter 1998 and rested summer 1998. For ease of interpretation and to maintain continuity, those results will be repeated here in context with the hyperbaric treatment.

The ambient water temperature for the winter acclimated mullet was 12.1 ± 0.1 °C and were therefore stored at 6.0 ± 0.1 °C. For summer acclimated mullet the ambient temperature was 20.1 ± 0.1 °C and were stored at 10.0 ± 0.1 °C.

7.3.4 White muscle pH measurement and metabolite determinations

Cut-surface WM pH measurements and WM samples were taken from the winter acclimated fillets prior to going into storage and then after ~12, 27, 37 and 50 h and in summer acclimated mullet ~12, 27, 37, 50 and 57 h after introduction of the anaesthetic. Lactate, ATP, P_i, creatine and glycogen were measured in the WM samples with methodology as described in Chapter 2.

7.3.5 White muscle mitochondria yield measurements

Experiments on mitochondria (Chapter 6) were carried out at the end of 1999 and throughout the year 2000. With every mitochondria extraction the yield (amount of mitochondria extracted from the WM) was measured (for methods: see Chapter 6: Section 6.3.6 Isolation of mitochondria from the white muscle). The relationship between the acclimated (ambient) seawater temperature and yield was plotted.

7.3.6 Statistical analysis

All times, pH values and biochemical values stated in the text are the mean ± standard error of the mean (SEM). Graphing and statistical analyses were performed using SigmaPlot 2000 for Windows Version 6.00 (Copyright 1986-2000 SPSS Inc.) and Microsoft® Excel 2000.

7.4 RESULTS

7.4.1 Post-harvest condition of winter and summer acclimated mullet

There was no difference in the weight and length of fish sampled in summer and winter, (Table 7.2) resulting in no difference in the CF, but winter fish had higher HSI consistent with glycogen storage in the liver prior to gonad development leading into summer. Winter acclimated fish took longer to reach anaesthesia than summer fish, reflected in the later sampling time. There was also no difference in blood pH and blood lactate between the two groups of fish (Table 7.2). There was no relationship between blood pH and sampling time, but there was a weak positive correlation between blood lactate concentration and blood sampling time when the data from summer and winter mullet were pooled (correlation coefficient = 0.62), with later sampling times corresponding to higher blood lactate concentrations (Fig. 7.1). This was attributed to the winter acclimated mullet possibly becoming hypoxic during anaesthesia as they were exposed to the anaesthetic for longer. There was no difference in the mean blood glucose concentrations of the two groups of fish (Table 7.2).

The initial cut-surface pH of winter WM was significantly higher than in the summer WM (Table 7.3) with samples being taken later than summer acclimated fish. The winter acclimated WM also had significantly lower levels of creatine and P_i. Pre-storage lactate, ATP and glycogen levels were similar between summer and winter acclimated fish (Table 7.3).

7.4.2 Post-mortem white muscle pH profiles

The decline in PM muscle pH in the summer and winter acclimated fish is shown in Fig. 7.2. In WM held under normobaric conditions the decrease in pH with time occurred at a similar rate between winter and summer acclimated mullet with the ultimate pH being reached after 27 h (Fig. 7.2a).

Table 7.2. Size, condition and whole blood measurements from rested summer and winter acclimated mullet.

	Summer 1998 (n = 5)	Winter 1998 (n = 5)
Weight (g)	214.8 ± 23.3	176.5 ± 20.0
Length (mm)	247 ± 7	228 ± 9
CF[1]	1.40 ± 0.04	1.46 ± 0.01
HSI[2]	2.93 ± 0.41	4.26 ± 0.34*
Pithing time (h)	0.61 ± 0.04	0.95 ± 0.04*
Blood pH	7.72 ± 0.05	7.67 ± 0.08
Blood lactate (mmol/L)	1.7 ± 0.5	3.9 ± 0.9
Blood glucose (mmol/L)	6.3 ± 0.3	6.7 ± 0.8

[1]Condition factor (CF) = weight (g)/length (cm^3) x 100 (Love 1980).
[2]Hepatosomatic index (HSI) = liver weight (g)/weight (g) x 100 was (Love 1980).
Values are the mean ± SEM (standard error of the mean).
* Significantly different from summer fish ($P < 0.05$; Student's -test).

Table 7.3. Pre-storage white muscle metabolite levels in rested summer and winter acclimated mullet.

	Summer 1998 (n = 5)	Winter 1998 (n = 5)
Sampling time (h)	1.13 ± 0.12	1.69 ± 0.18*
White muscle pH	7.61 ± 0.02	7.74 ± 0.03*
Lactate (μmol/g)	12.7 ± 1.0	10.0 ± 0.5
ATP (μmol/g)	7.4 ± 0.7	6.1 ± 0.7 n=3
P_i (μmol/g)	22.9 ± 2.1	13.7 ± 1.2*
Creatine (μmol/g)	12.6 ± 1.0	8.6 ± 0.6*
Glycogen (μmol/g)	37.6 ± 5.6	43.2 ± 1.8

Values are the mean ± SEM (standard error of the mean).
Significantly different from summer fish ($P < 0.05$; Student's -test).

Although the initial WM pH was higher in the winter acclimated fish, under hyperbaric conditions the pH decreased at such a rate PM that the ultimate pH was reached after 37 h, ~13 h before the hyperbaric summer acclimated WM (Fig. 7.2b). The profile of PM cut-surface pH change in the winter acclimated WM held under hyperbaric conditions was similar to summer acclimated WM held under normobaric conditions (Fig. 7.2b).

7.4.3 Post-mortem white muscle metabolite profiles

Lactate

There was no difference in lactate concentration over the first 12 h of PM storage of summer and winter acclimated mullet WM held under normobaric conditions (Fig. 7.3a). After 12 h storage the lactate concentration rose in both treatments with the summer acclimated fish WM reaching a higher level than the winter WM (~75-80 μmol/g muscle mass and ~60 μmol/g muscle mass, respectively).

There was a ~12 h delay in accumulation of lactate in the WM of both summer and winter acclimated mullet stored under hyperbaric conditions (Fig. 7.3b). The delay being more pronounced in summer WM. Following the delay the lactate concentration in the summer WM rose abruptly and continued to rise to 75.5 ± 2.0 μmol/g muscle mass over the remaining storage period (57 h). In contrast, lactate concentration in the hyperbaric winter only rose to 52.3 ± 1.2 μmol/g muscle mass after 50 h storage (Fig. 7.3a). However, it was possible that further lactate production (reaching ~60 μmol/g muscle mass) could have occurred in the winter acclimated hyperbaric treatment if storage had continued past 50 h (see normobaric fish, Fig. 7.3a).

The relationship between WM pH and WM [lactate] for summer and winter acclimated mullet held under normobaric and hyperbaric conditions in shown in Figs. 7.4a & b, respectively. Under normobaric conditions there was no difference in the relationship between summer and winter acclimated mullet (Fig. 7.4a). Under hyperbaric conditions the pH/lactate relationship in the summer WM was similar to the normobaric tissue, however, the relationship in the winter acclimated mullet was quite different. The WM pH in the hyperbaric winter preparation reached the ultimate pH while the WM [lactate] was still relatively low (Fig. 7.4b).

ATP

The depletion of ATP in the WM during PM storage under normobaric and hyperbaric conditions is shown in Figs 7.5a & b, respectively. The profiles for ATP depletion in WM held under normobaric conditions were similar in the winter and summer acclimated fish (Fig. 7.5a). The concentration of ATP in the WM decreased rapidly and reached low levels (<1 μmol/g) after 27 h storage. The profile of ATP depletion was also similar in winter and summer acclimated WM held under hyperbaric conditions (Fig. 7.5b). The level of ATP in the winter WM was generally lower than in summer WM, however, this was not statistically significant until 27 h storage. Low levels of ATP in the WM of both summer and winter acclimated fish were reached towards the end of the storage period.

Inorganic phosphate

P_i accumulation in the WM of summer and winter acclimated fish held under normobaric and hyperbaric conditions is shown in Figs. 7.6a & b, respectively. There was a significant difference between the immediate post-harvest WM P_i concentration in the summer and in the winter acclimated fish. In WM held under normobaric conditions (Fig 7.6a), even though there was the initial difference in P_i levels in summer and winter fish after 12 and 27 h storage, the levels were not significantly different in the two groups. After 27 h the P_i concentration in summer acclimated WM rose to ~45 μmol/g muscle mass, whereas in winter WM the P_i level reached a plateau at ~33 μmol/g muscle mass. There was no difference in the total accumulated concentration of P_i under normobaric storage conditions (summer increase 19.0 ± 3.1 μmol/g muscle mass; winter increase 20.4 ± 1.7 μmol/g muscle mass).

Summer acclimated WM held under hyperbaric conditions did not show an increase in P_i levels until after 27 h storage (Fig. 7.6b). This was consistent with the delay in ATP depletion (see Fig. 7.5b). After 27 h the P_i level then rose sharply to peak at ~45 μmol/g muscle mass. In contrast, P_i concentrations in winter acclimated WM held under hyperbaric conditions showed an immediate increase during storage, although the maximum rate of accumulation was much slower than in summer WM and the highest level reached was only ~30 μmol/g muscle mass. Although the initial [P_i] in summer acclimated mullet WM was ~10 μmol/g muscle mass higher than in winter

WM, the overall increase in P_i during hyperbaric PM storage was significantly greater in summer WM (summer increase 22.8 ± 2.1 μmol/g muscle mass; winter increase 13.6 ± 1.2 μmol/g muscle mass; $P < 0.01$, Student's t-test).

Creatine

Levels of creatine in the WM of summer and winter acclimated fish held under normobaric and hyperbaric conditions are shown in Figs 7.7a and b, respectively. The data set has been truncated due to the unreliability of measurements over 20 μmol/g muscle mass (See Chapter 2: Biochemical methodologies). Pre-storage levels of creatine in the WM of summer acclimated mullet were higher than in winter WM. In WM held under normobaric conditions (Fig. 7.7a) the creatine concentration of the WM in both summer and winter acclimated fish rose immediately during PM storage with summer acclimated WM creatine rising to over 20 μmol/g muscle mass and winter WM reaching ~18 μmol/g muscle mass. During PM storage creatine levels in the WM of summer acclimated fish were always higher than in winter fish. As discussed in Chapter 3, there was a problem with the creatine assay that resulted in levels above 20 μmol/g muscle mass being under-estimated. Thus, it is possible that the creatine levels in the WM of summer acclimated fish may have reached higher levels than ~23 μmol/g muscle mass. As the creatine concentration in the WM of winter acclimated fish was below 20 μmol/g muscle mass it was thought that these were accurate. There was no significant difference in the net increase in concentration in creatine under normobaric storage conditions (Fig. 7.7a). The summer acclimated WM increased to 14.8 ± 2.7 μmol/g muscle mass (assuming an initial level similar to the winter initial level) while the winter acclimated muscle increased to 19.0 ± 3.1 μmol/g muscle mass.

In summer acclimated WM stored under hyperbaric conditions there was a drop in the concentration of creatine in the WM after ~12 h storage (Fig. 7.7b). It was possible that creatine phosphate may have been generated over the first 12 h of hyperbaric storage reducing the free creatine concentration in the WM. But there was no fall in P_i or ATP making this possibility unlikely. Due to the minimal variation in the creatine measurement it is more probable to be the heterogeneity of the WM sample that produced the differences in concentrations. Thus, over the first 27 h of storage there was little difference in WM creatine concentration between summer and winter acclimated mullet stored under hyperbaric conditions. After this time creatine levels in

winter acclimated fish reached a plateau at ~18 μmol/g muscle mass whereas in summer acclimated fish the concentration continued to rise to over 20 μmol/g muscle mass. Paralleling the differences in P_i levels the final creatine concentration achieved during hyperbaric PM storage was significantly greater in the summer acclimated WM.

Glycogen

The changes in concentration of glycogen (as glucosyl units) in the WM of summer and winter acclimated mullet held under normobaric and hyperbaric conditions are shown in Fig. 7.8a and b, respectively. Although the glycogen assay produced quite variable results (particularly in the summer acclimated WM) some clear trends could still be seen. There was no difference in pre-storage levels of glycogen in the WM of summer and winter acclimated mullet (~40 μmol/g muscle mass). In WM stored under normobaric conditions, glycogen levels in summer acclimated fish remained high over the first 27 h storage and then dropped sharply and remained at low levels (Fig. 7.8a). In the WM from winter acclimated mullet the glycogen concentration dropped to 10 μmol/g muscle mass after only 12 h storage (Fig. 7.8a). In summer acclimated WM stored under hyperbaric conditions glycogen levels remained constant over the first 27 h storage (Fig. 7.8b). This was followed by a rapid decline down to 12 μmol/g muscle mass over the remaining storage period. In winter acclimated WM there was an initial decrease in glycogen over the first 12 h. The glycogen level did not change over the next 15 h storage, and then decreased to ~10 μmol/g muscle mass after 50 h storage (Fig. 7.8b).

7.4.4 Acclimation temperature and white muscle mitochondria yield

The relationship between acclimation temperature and WM mitochondria yield is shown in Fig. 7.9. Although there was a lot of variation in the yield measurement between different fish at the same temperatures the highest yields of mitochondria were extracted from the WM when the acclimated temperature was between 13 and 17.5 °C. Above this temperature the yield tended to be lower. At temperatures below 13 °C the yield was very poor and at such low yield levels mitochondria respiration experiments could not be carried out.

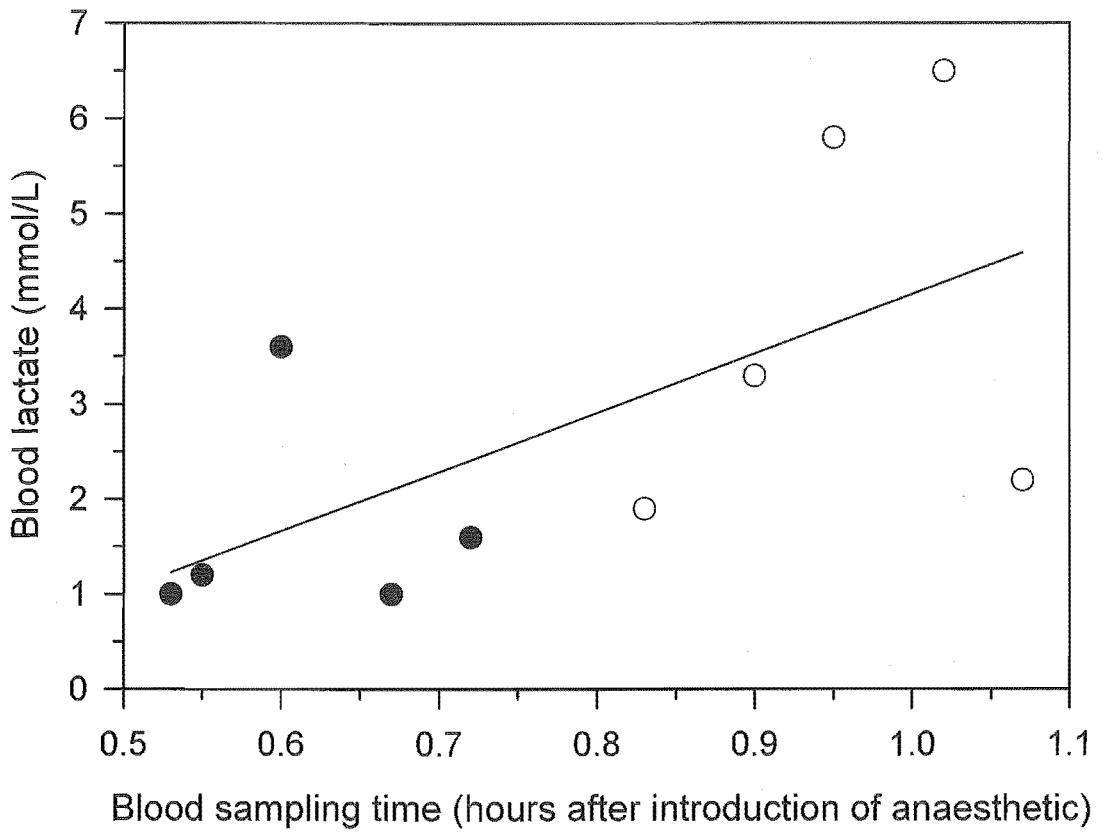

Figure 7.1: *Relationship between blood lactate concentration and blood sampling time in yellow-eye mullet acclimated to summer (20.1 °C ●) or winter conditions (12.1 °C ○). The relationship is described by the linear regression equation* $y = 6.22x - 2.07$, $r^2 = 0.39$, *and has a correlation coefficient of 0.62.*

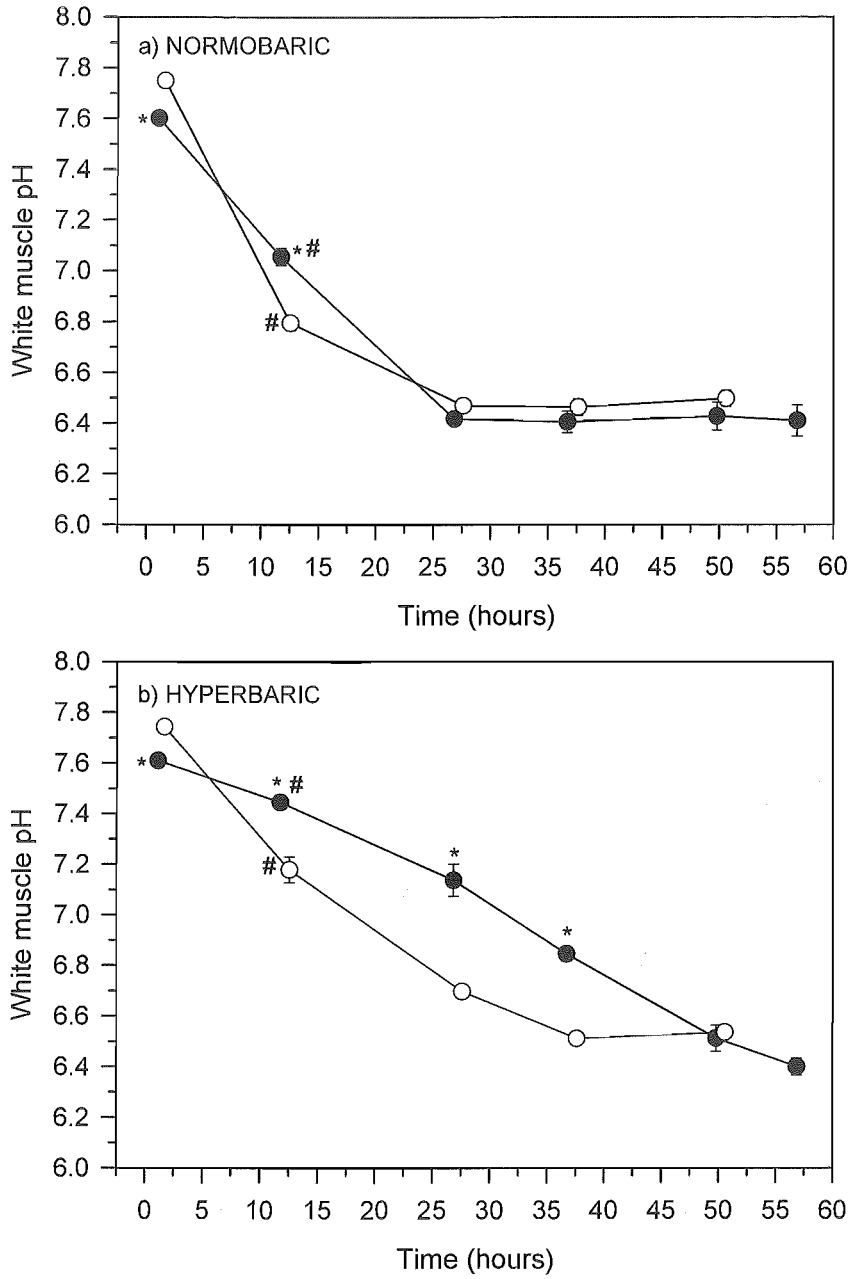

Figure 7.2: *Post-mortem cut-surface pH of epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions (ambient pressure, oxygen flow of 50 mL/min ± 1.25%) or b) hyperbaric conditions (620 ± 10 kPa, oxygen flow 50 mL/min ± 1.25%). Mullet were sampled during either summer (●), or winter (○). White muscle was stored at half the acclimated temperature of the fish (6.0 ± 0.1 °C) in winter (ambient temp. 12.1 °C) and 10.0 ± 0.1 °C in summer (ambient temp. 20.1 °C). Values are the mean ± SEM, n = 5. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

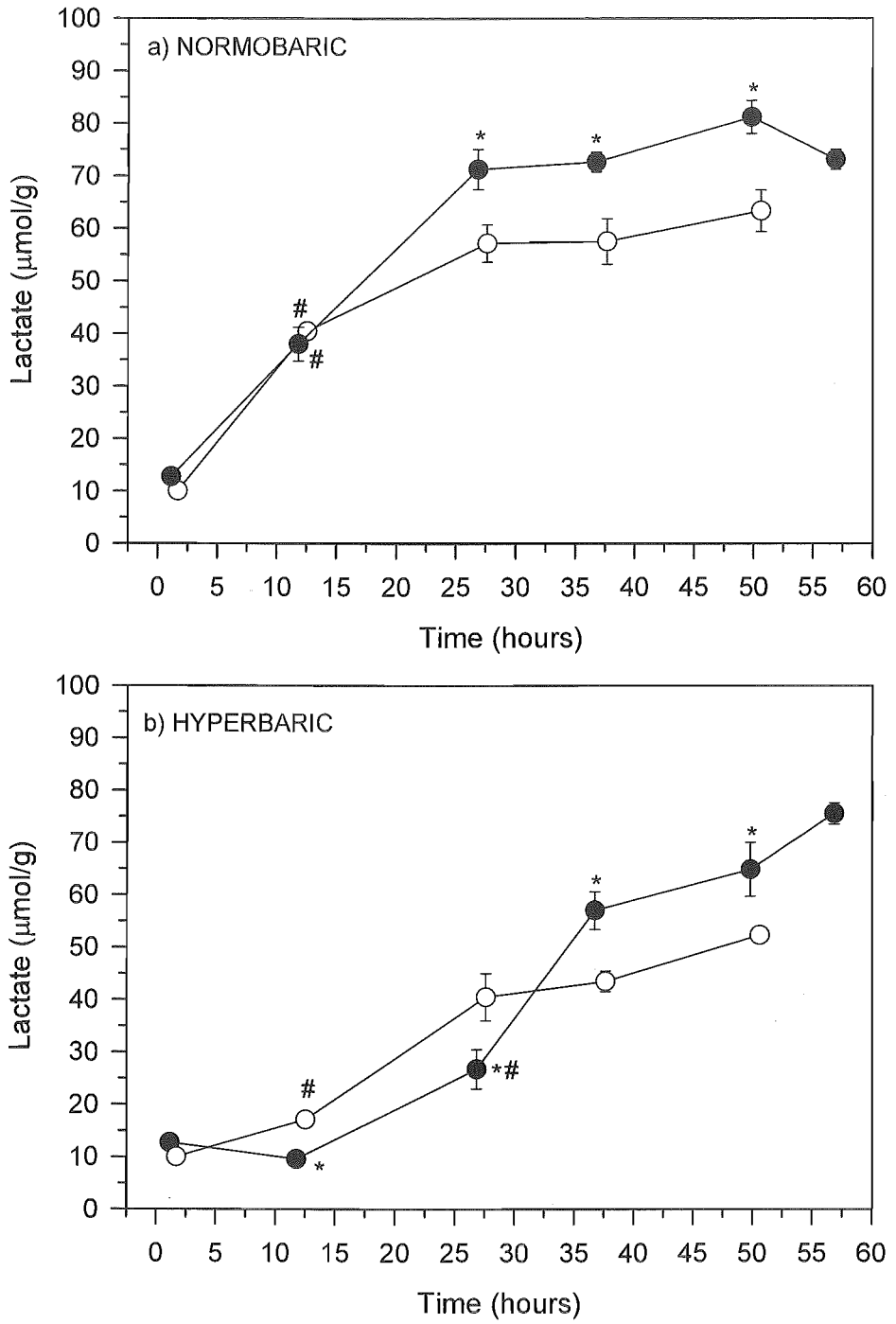

Figure 7.3: *Post-mortem [lactate] of the epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions or b) hyperbaric conditions. Mullet were sampled during either summer (●), or winter (○). Storage details as for Fig. 7.2. Values are the mean ± SEM, n = 5. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

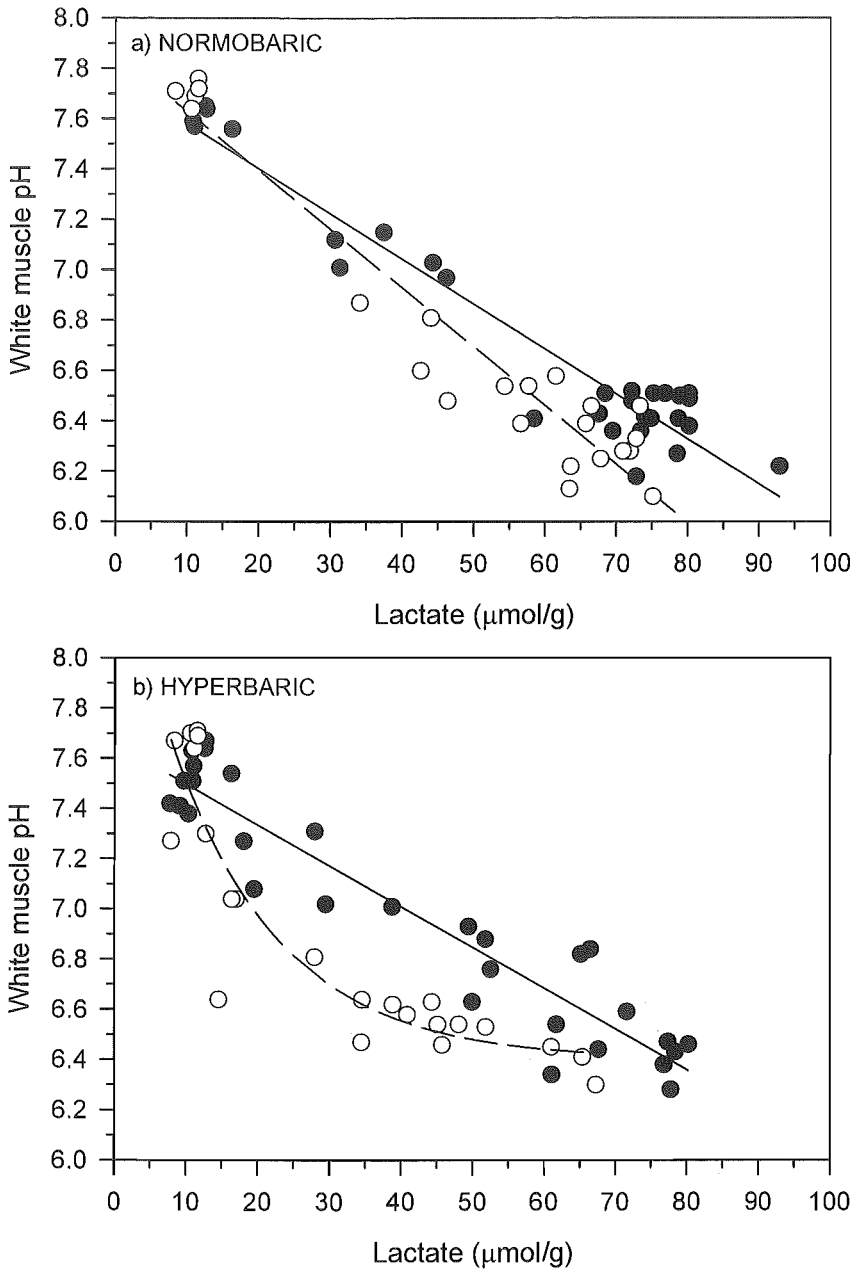

Figure 7.4: *Relationship between post-mortem pH and [lactate] from the epaxial white muscle of the yellow-eye mullet. The pH/[lactate] relationship of white muscle held under a) normobaric conditions from summer acclimated mullet (●, solid line) is described by the linear regression equation* y = *-0.018x + 7.76,* r^2 = *0.93; and for winter acclimated white muscle (○, dashed line) described by the linear regression equation y = -0.023x + 7.86,* r^2 = *0.93. The pH/[lactate] relationship of white muscle held under b) hyperbaric conditions from summer acclimated mullet (●, solid line) is described by the linear regression equation* y = *-0.016x + 7.66,* r^2 = *0.90; and for winter acclimated white muscle (○, dashed line) described by the exponential regression equation* $y = 6.40 + 2.14e^{-0.07x}$, r^2 = *0.82.*

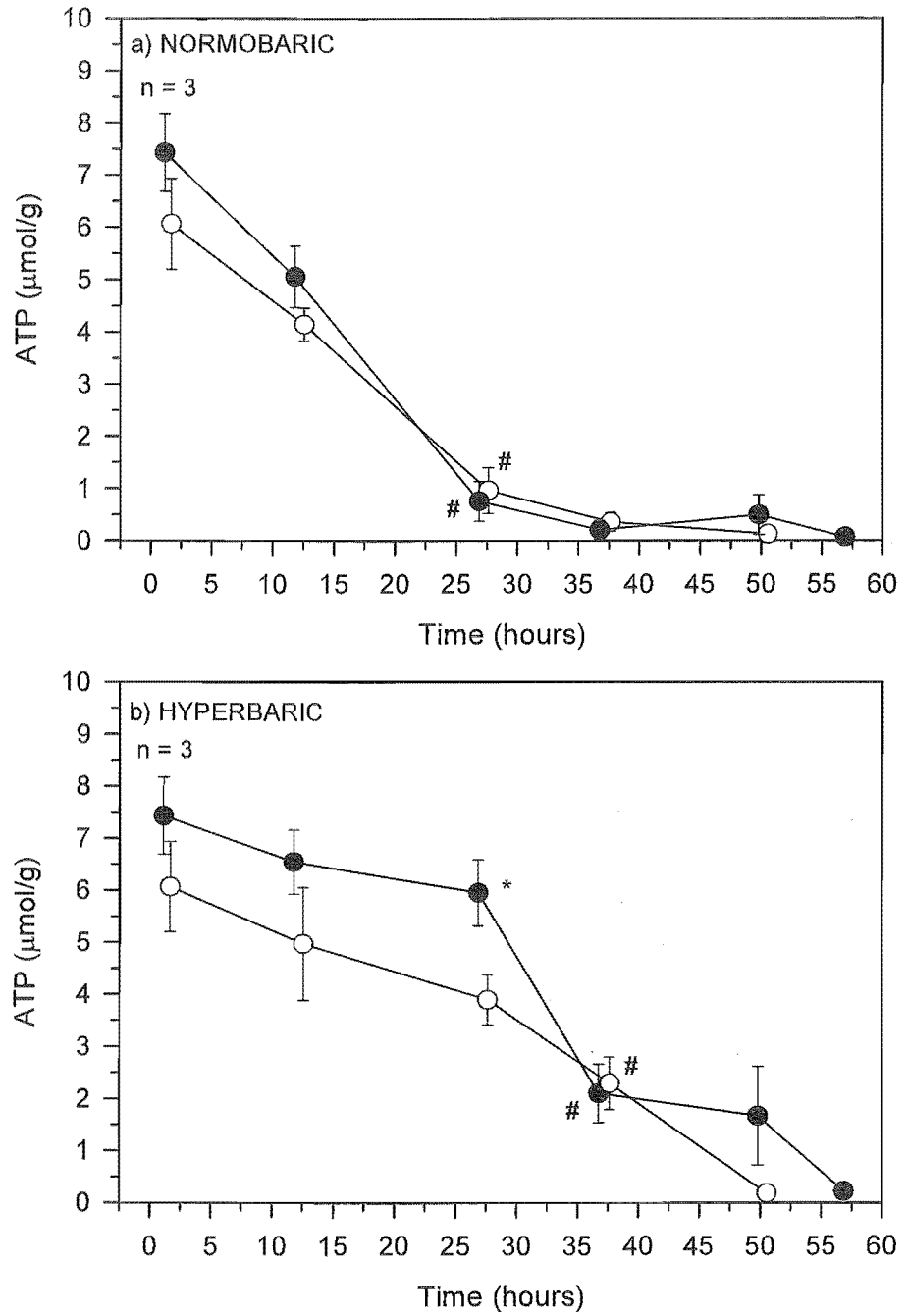

Figure 7.5: *Post-mortem [ATP] in the epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions or b) hyperbaric conditions. Mullet were sampled during either summer (●), or winter (○). Storage details as for Fig. 7.2. Values are the mean ± SEM, n = 5. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

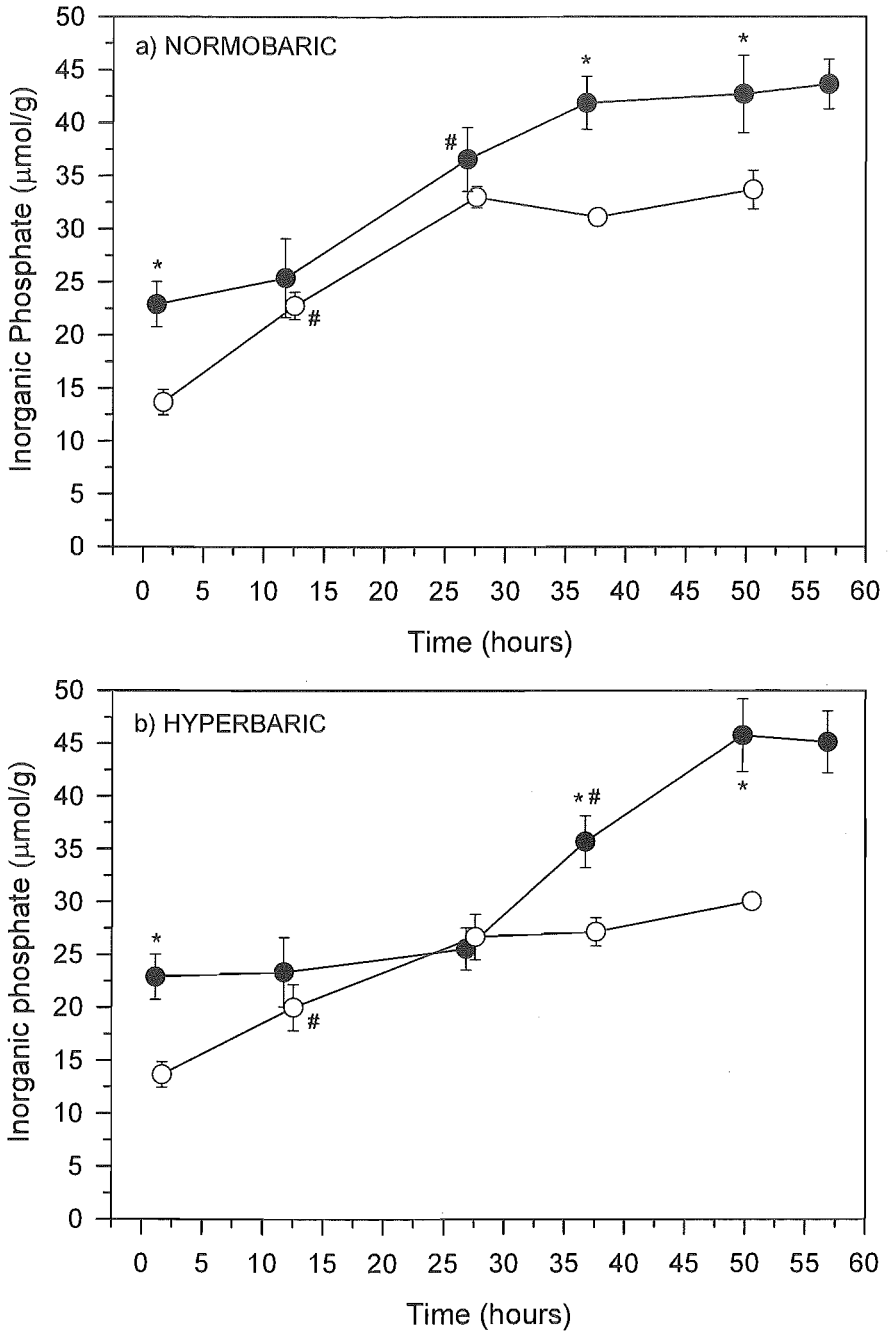

Figure 7.6: *Post-mortem [P_i] in the epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions or b) hyperbaric conditions. Mullet were sampled during either summer (●), or winter (○). Storage details as for Fig. 7.2. Values are the mean ± SEM, n = 5. * indicates a significant difference (P<0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P<0.05).*

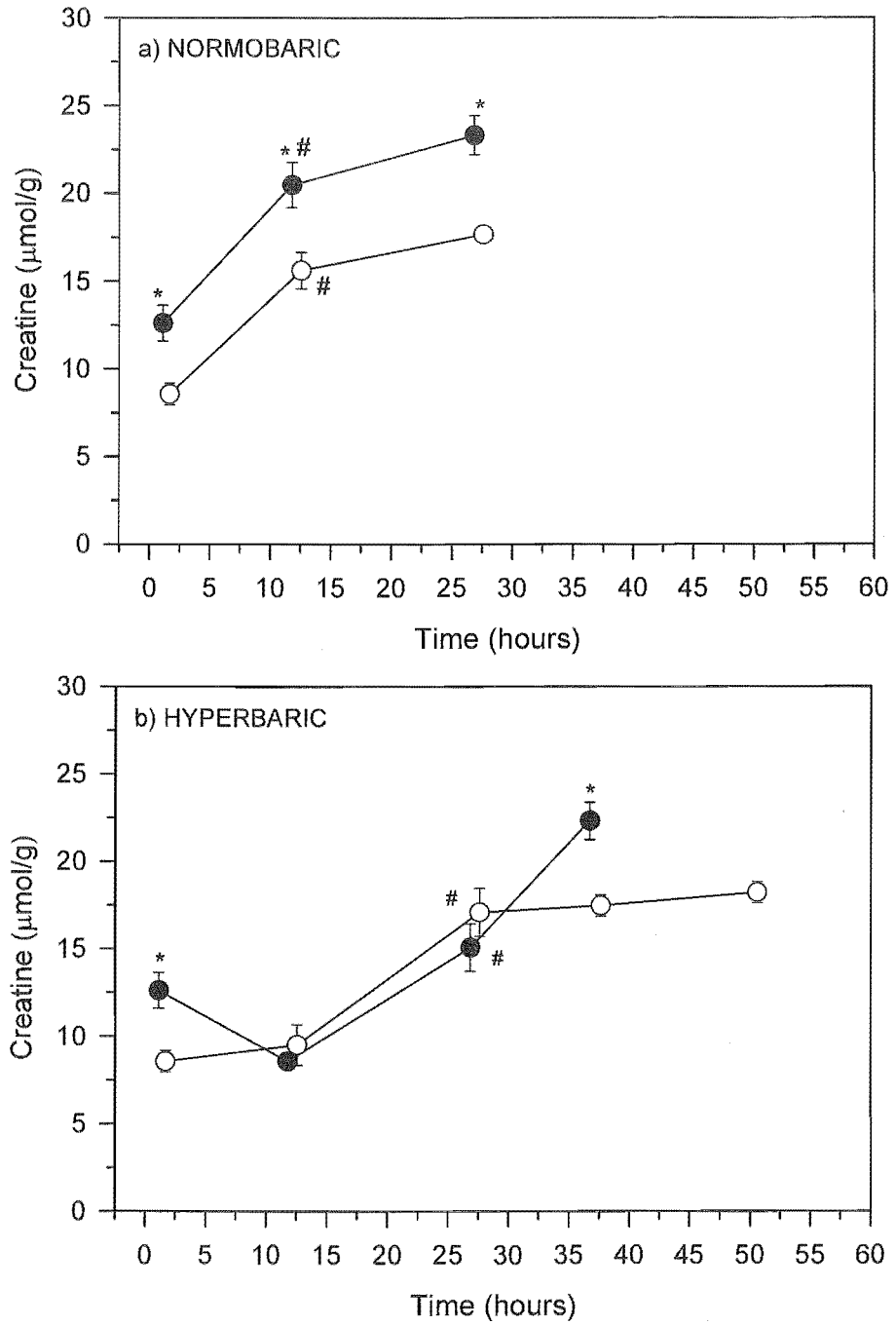

Figure 7.7: *Post-mortem creatine content in the epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions or b) hyperbaric conditions. Mullet were sampled during either summer (●), or winter (○). Storage details as for Fig. 7.2. Values are the mean ± SEM, n = 5. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

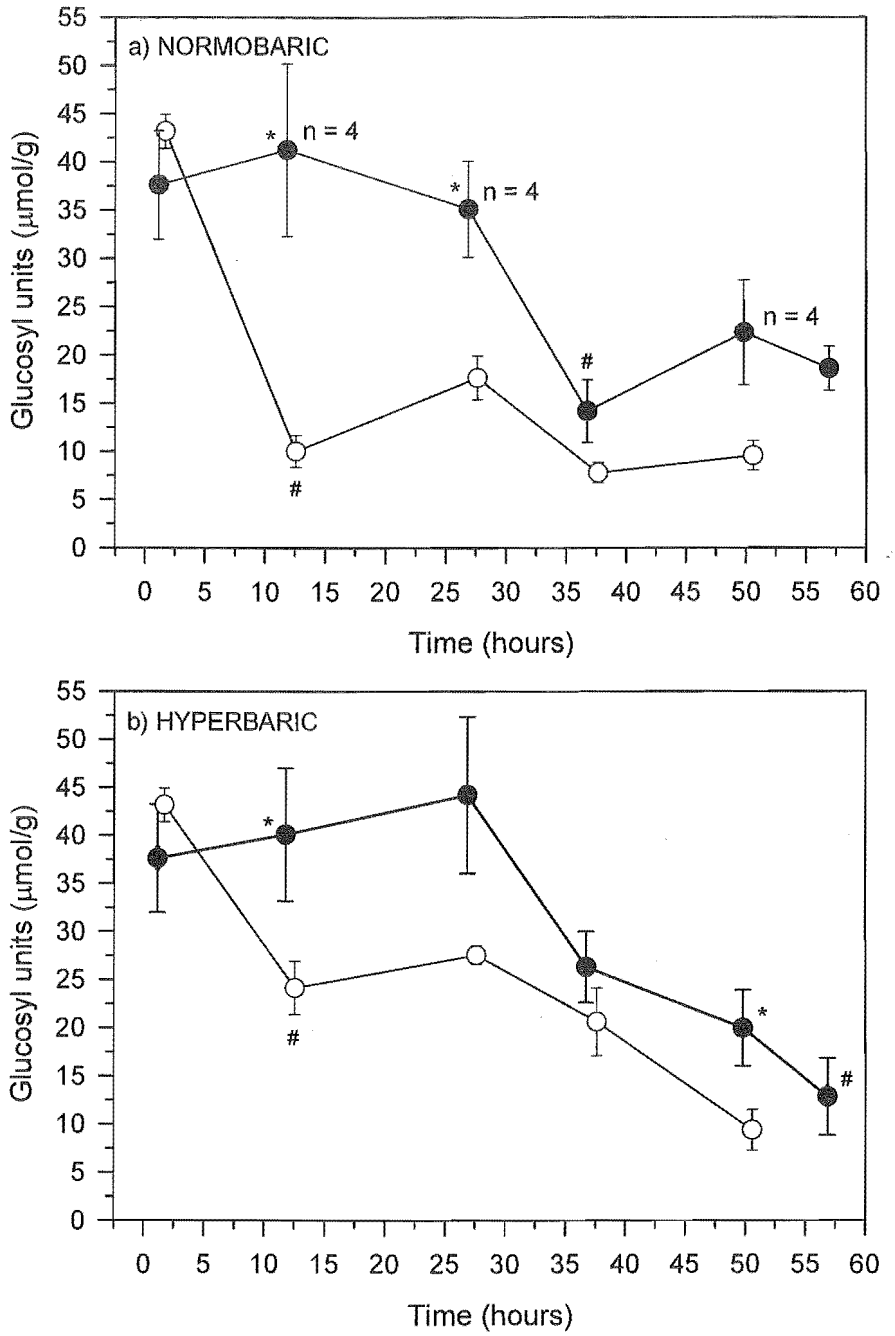

Figure 7.8: *Post-mortem glycogen content (as glucosyl units) in the epaxial white muscle of rested yellow-eye mullet stored under a) normobaric conditions or b) hyperbaric conditions. Mullet were sampled during either summer (●), or winter (○). Storage details as for Fig. 7.2. Values are the mean ± SEM, n = 5 unless otherwise stated. * indicates a significant difference (P < 0.05 Student's t-test) compared with the winter acclimated value at the corresponding sample time. # indicates a significant difference from the initial pre-storage value – only first instance marked (Sign test; P < 0.05).*

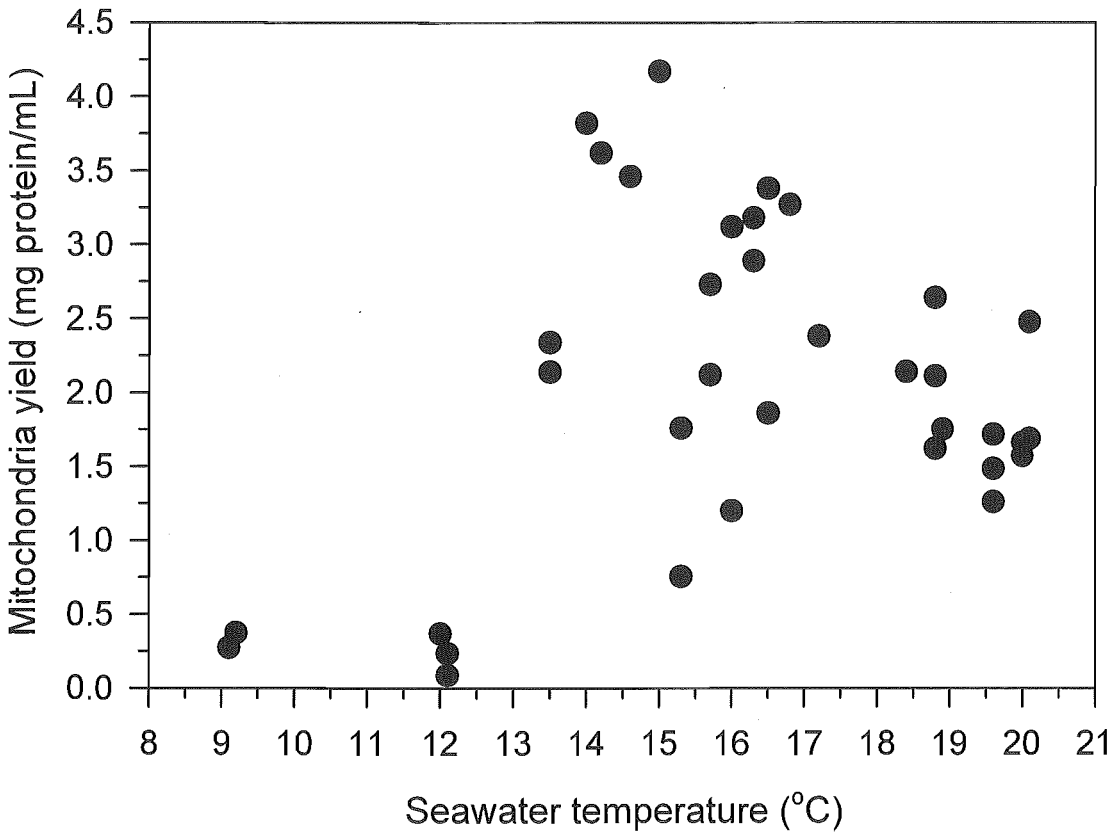

Figure 7.9: *Relationship between post-mortem mitochondria yield from the epaxial white muscle of the yellow-eye mullet and ambient seawater temperature. Each point represents one fish.*

7.5 DISCUSSION

7.5.1 Methodology

Although several studies have been carried out to determine the effect of acclimation temperature on the progression of rigor mortis during PM storage, they have not been carried out at previously determined optimum storage temperatures (Abe & Okuma 1991; Hwang et al. 1991; Watabe et al. 1990). The optimum PM storage temperature for rested yellow-eye mullet WM had been previously determined at this laboratory (Law & Jerrett 1996, unpublished results; Chapter 3: Fig 3.1).

The three studies mentioned above (Abe & Okuma 1991; Hwang et al. 1991; Watabe et al. 1990) did not use rested harvesting techniques and used very small sample numbers (sometimes n = 1). There would have been substantial handling artefacts in these fish resulting in high variation in physiological response to the handling and storage treatment. High variation together with low sample numbers meant that some of the conclusions made from the results may have been misleading. In the current study small sample sizes (n = 5) were used at each acclimation temperature but the WM had been well characterised during PM storage and there was little variation in the WM pH measurement. In addition, each fish had essentially its own control to the treatment with the LHS fillet stored under ambient conditions and the RHS stored under hyperbaric conditions. Generally, the differences between treatments were obvious, despite the small sample sizes. This was attributed to careful rearing and controlled, rested harvesting prior to post-harvest storage.

Mullet used in the winter acclimated treatment were sampled in September 1998 and this could be classed by some as spring sampling. However, daily ambient seawater temperature records kept at the laboratory over the last 8 years suggest that the mean acclimated temperature of 11.5 ± 0.1 °C (± SEM) 14 days prior to sampling was still within the typical winter temperature range. The underlying assumption of this chapter was that the main effect was due to seasonal temperature changes and not changes cued by other seasonally varying factors such as photoperiod. This will require further investigation to determine the effect of a combination of such environmental factors on the metabolism of the WM.

7.5.2 Physiological and behavioural adaptation to cold temperatures

Even though yellow-eye mullet are active during the winter they are noticeably more sluggish than during summer months (personal observation). The fish held at this laboratory are routinely fed to satiation with a sharp drop in food consumption below ambient seawater temperature of ~13 °C (Jerrett & Coubrough 2002, unpublished results). During winter the productivity of plankton in seawater is at its minimum, with low light levels and low temperatures decreasing photosynthesis (Paul 2000) generally depressing the amount of feed available for these estuarine detritus feeders. To cope with the reduced energy intake natural compensatory mechanisms must occur to reduce their activity level. One such mechanism in teleost tissues is at low temperatures (i.e. winter) the contraction time of muscle is slower and the maximum tail-beat frequency is also reduced compared with summer temperatures (Wardle 1980) thus the fish swim slower.

Mullet also reside deeper in the water column during the winter months (personal observation), possibly to avoid predation from birds at the surface who are also food-deprived over winter. As the mullet cannot swim as fast at the lower temperatures this is an appropriate behavioural strategy to avoid aerial attacks. Mullet held in indoor tanks during the current study also showed some of these behavioural changes such as reduced appetite and lower activity levels. This reduction in physiological activity (and therefore energy demand) may represent a strategy exhibited by mullet which favours survival in unfavourable environmental conditions. The mullet may conserve their energy reserves until the next period of high productivity allowing them to inhabit a wider temperature range.

7.5.3 Post-harvest differences in winter and summer mullet

Physical condition

Although fish were of the same size and condition (See Table 7.2) the HSI in winter fish was significantly higher than in their summer counterparts. The larger livers in winter fish could be indicative of a higher level of glycogen storage. However, increased liver size due to lipid deposits is also a possibility and requires further investigation.

Mullet sampled during winter took significantly longer to reach anaesthesia than the summer fish (Table 7.2). As discussed in Section 7.5.2, the tank-reared mullet were noticeably more sluggish during the winter months. Any concomitant slowing in respiration with lower winter temperatures could reduce the rate at which the anaesthetic (AQUI-S^{TM} Plus) was absorbed. This would then result in an increase in the time it takes the fish to become anaesthetised. This increase in time to reach anaesthesia in winter acclimated fish has also been observed in snapper (P. auratus, Jerrett et al. 2002).

Blood measurements

The blood pH for the winter rested mullet (1998) was slightly lower, but not significantly to that measured in the rested summer mullet (Table 7.2). As discussed in Chapter 3 (Section 3.5.5) studies investigating the effects of temperature on acid-base regulation in fish have shown that blood pH changes with temperature and has a linear relationship with a slope of typically –0.015 pH units/°C. Therefore the blood pH in the rested winter acclimated mullet should have been higher than that measured in summer fish. The discrepancy is most likely explained by the higher blood lactate concentration in the winter mullet. This suggested that the fish may not have been as rested as the summer fish resulting in a slightly depressed blood pH. There was also a positive relationship between blood lactate concentration and the time of sampling when the summer and winter data was pooled (Fig. 7.1). As sampling time increased blood lactate concentration also increased suggesting that some of the winter fish sampled after ~1 h after the introduction of anaesthetic were starting to become hypoxic.

The lower blood pH and higher blood lactate levels in the winter fish did not correspond with a lowering of the WM cut-surface pH in the winter mullet immediately after harvest or an increased level of lactate in the WM. In fact, from the general effect of temperature on pH (Schmidt-Nielsen 1992) it would be expected that the WM pH of winter acclimated fish be higher than in summer fish and this was indeed the case. The pre-storage cut-surface pH in winter WM was significantly higher than in summer WM (Table 7.3). Although the initial WM pH of winter WM was higher than summer WM this did not appear to confer an advantage during PM storage (Fig. 7.2b, see section 7.5.4 Post-mortem differences in winter and summer mullet).

It was unfortunate that an experiment to determine the effect of pre-mortem exercise on the WM was not carried out on summer acclimated mullet to allow a comparison with the winter exercised fish. However, from a study by Kieffer et al. (1994) showing the effect of environmental temperature on metabolic acid-base responses in rainbow trout after exercise it could be expected that summer exercised mullet would have a higher blood lactate concentration and metabolic proton concentration after exercise. Kieffer et al. also showed that rainbow trout acclimated to 18 °C had twice the blood [lactate] and [proton] after exhaustive exercise than trout acclimated to 5 °C.

Pre-storage white muscle metabolite concentrations

Although pre-storage WM [P_i] in the summer mullet was much higher than in the winter mullet the initial levels of ATP and glycogen in the WM were similar in summer and winter acclimated fish. As discussed in the Results it is also probable that the pre-storage WM creatine levels were also similar in summer and winter acclimated mullet. This gave little insight as to any metabolic differences between the fish acclimated to either summer or winter temperatures (Table 7.3). This was consistent with the findings of Kieffer et al. (1994) who reported that in rainbow trout ATP and glycogen content was similar in fish acclimated to either 18 or 5 °C. However, trout acclimated to 18 °C had higher PCr stores. Unfortunately PCr levels were not measured in the current study but the higher PM creatine levels measured in the summer mullet WM suggest that PCr stores may also have been higher (1:1 stoichiometry with Cr on hydrolysis) consistent with the results of Kieffer et al. (1994). Kieffer et al. also suggested that fish acclimated to warmer temperatures may have a greater capacity for immediate burst activity. In this study it was not until the changes in concentration of substrates and metabolites of WM were characterised during PM storage under hyperbaric, hyperoxic conditions that major differences were uncovered. This highlighted the utility of SOS style challenges to the WM in the discrimination of changes in metabolism.

7.5.4 Post-mortem differences in winter and summer mullet white muscle

White muscle metabolism

The cut-surface pH of hyperbaric summer WM did not fall below 7.0 until after ~30 h compared with ~17 h in winter WM (Fig. 7.2b). This difference suggested that the winter acclimated WM was not able to gain as much PM metabolic benefit from storage under hyperbaric conditions as the summer acclimated WM.

From the results of the summer acclimated fish, the reduction in the rate of pH decline during PM storage and the delay in lactate and P_i accumulation and ATP depletion strongly suggested a period of aerobic metabolism at the start of the PM storage period. Even when anaerobic metabolism dominated and there were increases in WM lactate and decreases in WM ATP, the rate of pH decline did not increase, suggesting that glycolytic reactions had been retarded. In the winter acclimated hyperbaric WM there also appeared to be a period of aerobic metabolism (delays in changes to [lactate], [ATP] and [P_i]) but interestingly pH changes did not reflect this.

There were minimal differences in PM metabolite profiles between summer and winter and this was consistent with the results of Kieffer et al. (1994) who reported that acclimation temperature did not significantly affect the anaerobic capacity in rainbow trout. However, it may be difficult to resolve differences in the maximum anaerobic glycolytic rate with this preparation as the PM metabolic rate was typically a small fraction of the rate observed in fish WM during active contraction.

7.5.5 Possible alteration to white muscle buffering capacity

A question arose during experimentation in Chapter 3 as to whether the WM buffering capacity changed with summer and winter acclimation. In the current study it was found that there was a significant rise in WM [lactate] from the pre-storage level after 12 h storage in winter hyperbaric WM, compared with no increase in the summer WM. The rise in winter hyperbaric WM [lactate] was, however, not of the same magnitude as that in the normobaric WM (Fig. 7.3a & b). The data suggested that the buffering capacity in the hyperbaric WM of winter acclimated fish might be lower than in summer, producing a similar pH with correspondingly lower WM [lactate]. This was

indicated by the relationship between WM pH with lactate (Figs. 7.4a & b). There was a clear difference in the summer and winter WM stored under hyperbaric conditions. A lactate concentration of ~40 μmol/g muscle mass in summer mullet resulted in a WM pH of ~7.0. In winter WM the same lactate concentration was associated with a pH of ~6.6. Although there was not a significant difference in the pH/lactate relationship between summer and winter WM held under normobaric conditions (Fig. 7.4) the majority of the of the data points from the winter WM were below that of the summer WM. This was also supportive of the hypothesis that the buffering capacity of the winter WM was also lower than summer WM. The apparent reduced buffering capacity in the winter acclimated WM may also be attributed to the behavioural changes discussed in Section 7.5.2. As the mullet are thought to be less active in winter their burst activity would also be less and therefore their buffering capacity in the WM may not need to be as great. There may also be a link with diet. Because fish are poikilothermic the metabolic rate is directly correlated with water temperature (Schmidt-Nielsen 1990). With a decrease in temperature metabolic rate is decreased and growth rate is reduced (Burrows 1972). As mullet appear to reduce their food intake during winter months it could be expected that the amount and type of protein and amino acids in their WM may be altered. Imidazole-containing compounds, such as carnosine and anserine, are major contributors to the WM buffering capacity (Castellini & Somero 1981). Therefore it could be expected that during winter the concentration of some of these compounds may decrease due to reduced food intake, reducing the buffering capacity of the WM.

7.5.6 Post-mortem white muscle storage temperature sensitivity

Although the WM was stored at the optimum storage temperature for the acclimated temperature of the mullet (half the acclimated temperature), the absolute temperature change between acclimated and storage temperature was greater in the summer mullet (20.1 °C acclimated, 10.0 °C storage for summer; 12.1 °C acclimated, 6.0 °C storage for winter). This would represent a 100% potential Q_{10} change for summer acclimated fish and 60% of Q_{10} for winter acclimated fish. Therefore it could be expected that the summer WM would perform better than winter WM due to the greater Q_{10} effect of cooling on metabolic rate. However, there was little difference between the PM pH

profile in WM stored under normobaric conditions, suggesting that the glycolytic ability was not more compromised in the summer acclimated fillets. As discussed in the introduction, in winter acclimated mullet the temperature effect on PM muscle pH expressed as Q_{10} was 1.6 times greater compared with summer acclimated mullet, i.e. winter acclimated mullet were more sensitive to temperature change (Jerrett et al. unpublished data). In the current study, even though the absolute temperature difference between acclimated and storage temperature was lower in the winter acclimated mullet, it could be expected that the greater Q_{10} effect would result in minimal differences in the slowing of metabolic rate compared with summer acclimated fish.

7.5.7 Physical limitations of low temperature

It is possible that the environmental temperature had a significant influence on the rate of diffusion of metabolic end-products, such as CO_2, out of the WM. When winter WM was stored under hyperbaric, hyperoxic conditions there was an apparent period of aerobic generation of ATP but not to the same extent as summer WM. Any CO_2 produced during aerobic metabolism may not have been able to diffuse as rapidly out of the mitochondria and muscle cells of winter WM compared with summer WM. The possible increased level of CO_2 in the cells, in combination with acidification of the tissue could have disrupted oxidative phosphorylation, prematurely halting aerobic generation of ATP in the winter WM.

7.5.8 Possible alterations to substrate utilisation during post-mortem storage

Glycogen levels in fish generally decrease through the winter as a result of low food consumption (Moon & Foster 1995; Woo 1990). Woo (1990) also found that in red sea bream there was increased muscle lipid at cold temperatures along with increased glucose-6-phosphate dehydrogenase (G6P) activity. Increased activity in this enzyme was attributed to the provision of extra NADPH required for sustaining enhanced lipogenesis. While lipid was being created, carbohydrate would have been the dominant substrate for energy production. Similarly, in a study investigating fuel use during aerobic swimming at different temperatures in rainbow trout, it was shown that during winter (5 °C) carbohydrate was the most important fuel for swimming (Kieffer et al.

1998). However, in fish swimming at 15 °C lipid remained the most important fuel. In the current study differences in the ultimate concentrations of some substrates between summer and winter WM maybe consistent with these findings.

Although some of the initial levels of the WM metabolites measured were similar between summer and winter acclimated mullet, differences in ultimate concentration in the WM were evident. The discrepancy between final metabolite concentrations of P_i and creatine in hyperbaric winter acclimated hyperbaric WM suggested that the amount of PCr stored in the WM was lower in winter mullet. As the winter acclimated mullet were sampled later than summer mullet it was suggested that if the mullet had experienced hypoxia then some of the PCr stores may have been hydrolysed. This could have been the reason for the discrepancy in the final metabolite concentration of creatine, but the winter acclimated mullet WM had significantly lower pre-storage levels of creatine and P_i (Table 7.3). This, in turn, suggested that there was an absolute reduction in resting levels of PCr in the winter acclimated WM. Less PCr would result in less P_i and creatine after hydrolysis with the results from the current work supportive of this theory (see Figs. 7.6 & 7.7). This would directly lower the 'ATP potential' (term introduced by Bate-Smith & Bendall 1956) of the WM in winter acclimated mullet.

In the hyperbaric WM of winter acclimated mullet the [lactate] never reached the same levels as those measured in the summer acclimated mullet (Fig. 7.3b). Even though winter and summer acclimated mullet entered PM storage with similar levels of glycogen in the WM (~40 μmol/g muscle mass), under hyperbaric storage conditions glycogen levels in summer WM did not fall until after 27 h compared with ~12 h in winter WM (Fig 7.8b). This suggested that during the assumed period of aerobic metabolism the summer WM was able to use a substrate other than its stored glycogen for aerobic generation of ATP, sparing glycogen for anaerobic glycolysis. In the winter WM aerobic generation of ATP may have been fuelled only by the stored glycogen through activation of glycogenolysis. Therefore, in the winter acclimated WM glycogen may have become limiting, halting the production of lactate.

There was also a difference in final lactate levels in the WM of mullet held under normobaric conditions (~10 μmol/g muscle mass difference, Fig 7.3a). Glycogen levels in summer acclimated WM stored under normobaric conditions also showed a

~27 h delay before dropping (similar to summer hyperbaric WM) compared with winter WM where glycogen levels had dropped 10 μmol/g muscle mass after 12 h (Fig 7.8a & b). This again indicated that glucose for anaerobic glycolysis in summer WM was coming from a source other than the glycogen stores in the WM, resulting in the discrepancy between ultimate lactate levels. Overall, it would appear that the WM from summer acclimated mullet may have had more PCr stores in the WM and also did not have to rely solely on carbohydrate (glycogen) for aerobic and anaerobic generation of ATP as did the winter acclimated mullet. However, as lipid levels in the WM were not measured in the current study it is not certain that lipid was in fact used in the summer WM for aerobic generation of ATP.

A study by Kiessling et al. (1995) investigating the changes in WM energy metabolism during spawning of rainbow trout found that glycolytic capacity decreased to less than half whereas oxidative metabolism increased about two- to four-fold, with an increased capacity for fatty acid utilisation. If this were true for yellow-eye mullet which spawn in summer and autumn, lipid catabolism would be dominant in summer acclimated fish, with a greater reliance on carbohydrates in winter acclimated fish: a result also found by Woo (1990). These findings support the findings of the current study whereby WM from winter acclimated mullet stored under hyperbaric conditions showed a clear reliance on carbohydrate (glycogen) for ATP generation during PM storage compared with a possible "mixture" of both carbohydrate and lipid metabolism in summer acclimated mullet as described above. However, from the results there was no suggestion that glycolytic capacity had decreased in hyperbaric or normobaric summer acclimated WM as indicated by the Kiessling et al. (1995) study. Though this may not be resolved at the PM glycolytic fluxes measured in the rested PM WM.

7.5.9 Possible acclimation compensatory mechanisms in mullet

When comparing PM WM from winter and summer acclimated fish there was little difference in metabolic profile between the two when stored under normobaric conditions. It was only when WM was stored under hyperbaric conditions that the differences between the acclimation temperatures were clearly evident. If mullet followed a similar pattern in metabolic reorganisation during winter acclimation as other species (increase in the activities of mitochondrial enzymes, in mitochondrial

volume density or in the percentage of aerobic fibres in the swimming musculature; Table 7.1) then we could expect the WM to behave in a similar way during hyperbaric PM storage in both summer and winter acclimated mullet. However this was not the case as there were lower mitochondrial yields in the winter fish.

As mentioned in the Introduction, not all species of fish previously studied exhibit changes to aerobic capacity during winter. Rainbow trout (and presumably other salmonids) do not change their aerobic capacity in the WM as it is already high and therefore they do not need to increase it at low temperatures (Thibault et al. 1997). Salmonids are winter-active, mostly freshwater teleosts whereas yellow-eye mullet are not as active during the winter months (personal observation) and live in the marine environment. Compared with the winter active freshwater species, marine teleosts may not need to employ extensive cellular compensatory mechanisms to maintain their aerobic flux since energy expenditure has dropped (exercise less). In contrast to winter-active species, a study of the temperature adaptation responses of sea bass (winter-inactive marine teleost) by Trigari et al. (1992) found that there was an overall decrease of mitochondrial respiratory activity during cold acclimation in heart and liver. They also reported that there was no increase in unsaturated membrane lipids in response to cold (i.e. negative homeoviscous adaptation). They suggested that the limited ability of most marine teleosts to synthesise long-chain poly-unsaturated fatty acids (PUFA) may explain the differential response of sea bass and freshwater teleosts to low temperatures.

The mullet used in the current study were fed throughout the year even though their intake lowered over winter. It was therefore possible that the dietary intake of fatty acids (FA) in captive fish may have allowed a small increase in unsaturated of membrane lipid in response to cold. This may not be possible for fish in their natural habitat during winter.

Yellow-eye mullet may not be as active during winter as salmonids, however, they can not be classed as being as inactive as sea bass. Their activity levels during winter could place them at an intermediate level between the two. It was reasonable to suggest that the mullet may share some of the alterations to cellular metabolism that both winter active and winter inactive species employ. The reduced effect of hyperbaric PM storage in winter acclimated mullet WM compared with summer acclimated fish was consistent with an overall decrease in mitochondrial respiratory activity similar to

that observed in sea bass in response to cold. It was also consistent with the slightly reduced levels of ATP in the WM of rested mullet (Fig. 7.5a & b). If the rate of ATP synthesis was reduced over the winter due to a decrease in demand this could lower the overall level of ATP in the muscle. Even though oxygen was getting into the WM the likely lowered activity of the mitochondrial enzymes would mean that less ATP was able to be produced during the period of aerobic metabolism in the early stages of PM storage. The decrease in aerobic ATP supply would only leave one option: anaerobic glycolysis. Thus, even though the WM of winter acclimated mullet was able to respire aerobically at the start of PM storage the rate of ATP supply was not fast enough to satisfy the demand, resulting in a decrease in WM ATP levels. It was also possible that the WM of the winter acclimated mullet was already adapted to lower temperatures and, therefore, hypothermic PM storage had less effect on the WM, such that the metabolic rate of the tissue was actually higher than expected. As the metabolic rate of the WM was not directly measured in this study it was not possible to confirm which hypothesis held true.

The poor yield upon extraction of mitochondria from the WM of winter acclimated mullet also supported the hypothesis that mitochondrial respiratory activity may have been reduced. Due to the decrease in activity during winter the mullet may primarily rely on the RM for swimming, allowing the WM to decrease its numbers of mitochondria. It is also possible that mitochondria reduce in number during winter because growth rate is reduced, in turn, reducing the aerobic energy requirement in the WM, hence the poor yield on extraction.

Compensatory mechanisms and post-mortem storage

Studies investigating the progression of rigor mortis in carp at different acclimated temperatures have found that at cold acclimation temperatures the uptake of Ca^{2+} by the sarcoplasmic reticulum (SR) was faster than at warmer acclimation temperatures (Abe & Okuma 1991; Hwang et al. 1991; Watabe et al. 1989). Myofibrillar Mg^{2+}-ATPase activity was also increased with cold temperature acclimation. This increase in ATP consumption at cold temperatures was offered as an explanation of the faster onset of rigor mortis at low temperatures. It appeared that this may have been the case in winter acclimated mullet WM in the current study. Although Ca^{2+} uptake into the SR maybe

faster there only needs to be a very small increase in myofibril [Ca^{2+}] to activate the ATPase and accelerate ATP degradation (Watabe et al. 1990).

Jerrett et al. (2002) found that winter acclimated snapper (Pagrus auratus) WM was more sensitive to PM storage at low temperatures. The lower the acclimated temperature of the fish the more depressed the ultimate lower lethal limit (LLL) of the fish becomes. Woo & Fung (1980) found that the ultimate LLL of Chrysophrys major (similar to P. auratus) was 5.5 °C. This is close to the optimum storage temperature of P. auratus, a possible reason for its increased sensitivity to low storage temperatures. This is most likely the case in yellow-eye mullet as it has a similar optimum storage temperature to P. auratus, possibly a similar ultimate LLL, and also has increased sensitivity to low storage temperatures (Jerrett et al. unpublished data). Therefore with the storage temperature being close to the ultimate LLL and cellular membranes becoming increasingly permeable at low temperatures (Hochachka 1986; Hochachka et al. 1996) the increase in cytosolic [Ca^{2+}] could activate the ATPase hastening ATP degradation.

It is also important to remember that summer temperatures are also nearing the extreme of the fishes environment. A large proportion of the year is spent at seawater temperatures between 14 to 17 °C. Temperatures in this range are most likely to be the mullets optimum for growth, physiological and metabolic processes. Thus at acclimated temperatures of 20 °C the fish is closer to its ultimate upper lethal limit. Though this has yet to be determined for this species.

It was difficult to determine what cellular compensatory mechanisms occur at these higher temperatures as most studies (including the current study) compare summer and winter acclimated fish. The summer animals usually act as the control against which the changes in the winter animals are compared. It would, therefore, be of interest to instead compare the changes occurring in summer and winter acclimated fish to those acclimated to autumn/spring temperatures.

However, Jerrett et al. (2002) found that snapper (P. auratus) may only have two modes of "metabolic emphasis", i.e. a summer mode and a winter mode. If this is correct they would not continually adapt to increasing or decreasing temperatures. There appeared to be a temperature transition around 16 to 18 °C at which the metabolic changeover occurred. It was suggested that this strategy may reduce the relatively high

cost associated with thermal acclimation. It is possible that the same strategy is also present in mullet as they face a similar range in environmental temperature as snapper.

7.5.10 Questions to arise from these experiments

Although it was found that there was little difference in PM changes between summer and winter acclimated WM stored under normobaric conditions, the alterations that had occurred in the WM were clearly seen when stored under hyperbaric conditions. This storage method may be useful in resolving other changes to the WM whether they result from different acclimation temperatures or from changes in diet.

The clear differences seen under hyperbaric conditions give some insight into the problems associated with seasonal variation in commercial fish species. Further research concentrating on maintaining the PM "life" of the ischemic WM could also be better directed by using this method of storage to "test" the WM. Being able to target cellular mechanisms open to manipulation will result in a higher probability of success.

7.6 SUMMARY

During cold acclimation over the course of several weeks many species of fish undergo a series of compensatory biochemical modifications. Levels and activities of some glycolytic enzymes increase with cold acclimation along with an increase in mitochondrial volume density in red and white muscle. The maintenance of aerobic flux at cold temperatures is perceived to be of paramount importance. PM storage of WM under hyperbaric oxygen conditions resulted in a slowing of PM metabolic changes and an initial period of aerobic metabolism (Chapter 4). As cold acclimation leads to an increase in aerobic flux in some species it was hypothesised that cold acclimated fish would have a further increased period of aerobic metabolism when stored under hyperbaric conditions.

Yellow-eye mullet acclimated to summer temperatures and stored under hyperbaric conditions had a greater ability to respire aerobically during PM storage compared with mullet acclimated to winter temperatures. This, in turn, resulted in a decrease in the rate of PM substrate use and metabolite accumulation during hyperbaric storage in summer acclimated mullet. The biochemical changes suggested that winter acclimated WM had lower levels of PCr in the WM and relied primarily on carbohydrate for ATP generation compared with a possible mixture of both carbohydrate and lipid in summer acclimated WM. Summer acclimated WM had a greater apparent aerobic scope than winter WM with winter acclimated mullet possibly having a lower buffering capacity. Behavioural changes associated with cold acclimation in the mullet and further evidence from more winter inactive species can be correlated with these findings. Further work is needed to clarify if these hypotheses are, indeed, correct.

7.7 REFERENCES

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CHAPTER 8
General Discussion

Maintenance of living tissue out of its normal environment has been the focus of organ preservation researchers for several decades. The ability to extend the period of tissue viability when the organ is out of its normal functioning environment has proven difficult. Where an organ is well perfused by the circulatory system the we might expect that delivery of essential substrates and also removal of waste products would be easy to achieve. Trying to transfer technology developed for mammalian systems to a tissue where there is a very limited circulatory system (i.e. teleost WM) could be challenging. However, the isolated nature of the WM also confers possible advantages in terms of PM cell survival not available to well perfused tissues, in that the tissue's apparent isolation suggests self-sufficiency. The objective of the thesis was to investigate how cell viability can be maintained in rested ischemic WM during PM storage and the reasons why cell viability is eventually lost.

8.1 The significance of conserving energy stores

The maintenance of cell viability in an ischemic preparation, such as a WM fillet, requires a readily available energy supply. The duration of this viability will then be dictated primarily by the extent of the energy reserves available and their rate of utilisation. When fish are exercised prior to death their energy and substrate reserves (ATP, PCr, glycogen and fatty acids) can be depleted to varying extents. Their metabolic rate is increased which, in turn, promotes a widespread increase in the rate of all other chemical reactions, including the rate at which ATP is utilised (Bate-Smith & Bendall 1956). If the fish has been exercised prior to death, during PM storage of the ischemic WM it may have little, if any, ability to generate ATP to maintain cellular processes, such as the all important ion gradients. If glycogen is still available in the WM the only avenue for ATP generation is through anaerobic glycolysis. This is a process that acidifies the tissue it is generating energy for and so hastens the progression toward loss of cell viability and rigor mortis. The results from Chapter 3 showed that when fish were exercised prior to death there was also a large variation in the physiological disturbances that occurred in the blood and WM. Some fish displayed little disturbance to their acid/base balance and used few of their energy reserves,

whereas others depleted all their energy reserves and had large acid/base disturbances. This variation in response makes it extremely difficult to successfully manipulate the exercised PM muscle with a consistent outcome.

As shown in Chapter 3, using rested harvesting techniques to eliminate exercise prior to and during harvesting allowed the WM to retain its energy and substrate stores. There were also minimal physiological disturbances to the animal, including minimal acid/base balance disturbance in the blood and WM. The major advantage of this method of harvesting was the resultant consistency of the WM. This consistency extended to the biochemical profile of the WM during PM storage. Overall, there was a high level of confidence that the profile of PM changes occurring in the rested WM would be similar in all rested mullet WM.

Chapter 3 clearly showed that if the energy and substrate stores of the WM were retained during harvesting, the progression of PM changes and loss of cell viability occurring in the WM, was greatly retarded compared with WM that had been exercised prior to harvesting. This finding was consistent with the literature (Boyd et al. 1984; Jerrett et al. 1996, 1998; Law et al. 1997; Lowe et al. 1993; Thomas et al. 1999; Wells 1987).

8.2 Energy generation during post-mortem storage

Although in rested mullet ATP, PCr and glycogen levels in the WM were essentially intact prior to PM storage, the ischemic nature of the preparation meant that once the stored high energy phosphagens had been utilised the only option for ATP generation was by anaerobic means. This is a very inefficient process. As described above, anaerobic glycolysis also acidifies the WM and in the early PM is one of the main factors that over-extends the homeostatic mechanisms of the cell, eventually leading to cell death. Alternately aerobic respiration (oxidative phosphorylation) is the most efficient way to generate ATP using the substrates stored within the WM and potentially less disturbing to the maintenance of homeostasis in the PM than anaerobic glycolysis. Again, because the WM preparation was ischemic, delivery of oxygen to the WM cells was a major obstacle but still possible via direct diffusion as was the likely removal of some proportion of the CO_2 generated. In mammalian studies, including humans, it has been shown that hyperbaric oxygen (HBO) therapy increases the level of oxygen in the

blood promoting healing of damaged tissues. Therefore, if oxygen could be delivered to the rested WM for use in energy generation during PM storage the rate at which PM changes occur would be slowed or even halted. This was the focus of Chapter 4.

8.3 Modifying the post-mortem metabolism in the white muscle

A preliminary investigation showed that anaesthetised fish had a tendency to become distressed and exercise when manipulated under hydrostatic pressure. For both humane handling and in the interests of maintaining rested WM, the focus turned back to the ischemic WM preparation. Delivery of oxygen to the rested PM WM was achieved by storing the muscle under hyperbaric oxygen (assumed hyperoxic) conditions. Unlike the hyperoxic pre-loading experiments using the live fish, this preparation could be continuously exposed to the treatment during storage.

Increasing the pressure that the muscle was stored under decreased the rate at which the WM became acidified during PM storage. A flow of oxygen through the storage chamber while the muscle was stored under pressure was also critical to reducing the rate of PM acidification. It was thought that the flow of oxygen through the chamber eliminated waste products from the WM (e.g. CO_2), further delaying the acidification.

The hyperbaric, hyperoxic treatment appeared to offset the effects of low temperature stress on the PM WM. Normally temperatures lower than the optimum for benign hypothermia increase the rate of acidification in the PM WM. However, the WM pH profile during PM hyperbaric, hyperoxic storage did not change significantly. The benefit the WM gained from the assumed supply of oxygen during PM storage far out-weighed the deleterious effects of storage below the optimum storage temperature. When the WM was biochemically characterised during PM storage it became clear that the WM cells were able to generate ATP aerobically for a period of ~12 to 27 h. Endogenous ATP levels in the WM did not begin to drop until after 12 h PM storage with accumulation of lactate and P_i being delayed for a similar period. The same pattern was observed when the investigation was extended to two commercially important New Zealand species (snapper and chinook salmon; Chapter 5) stored under

hyperbaric conditions. When snapper WM was stored under hyperbaric, hyperoxic conditions the delay in ATP depletion and onset of anaerobic metabolite accumulation was extended to ~35 h. However, when chinook salmon (O. tshawytscha) WM was stored under hyperbaric conditions the aerobic period during PM storage was only ~12 h. It was suggested that the difference in structure of the WM and the strategies for recovery from burst exercise between the species (particularly the mullet and snapper) determined how beneficial PM storage under hyperbaric conditions could be. It was possible that snapper WM has a lower metabolic rate than salmon and mullet and also a lower mitochondria capacity resulting in slower PM changes in the WM. The tolerance for accumulated waste, the resting metabolic rate and "duty cycle" of the muscle all may contribute to the PM survival of rested tissue. Therefore, it would be of particular interest to extend the investigation further to those species that recover very slowly from burst exercise (e.g. benthic species) if the benefits gained from hyperbaric storage are related to the rate of recovery. Although the structure and function of the WM may play a role in PM metabolism it was also suggested that the physical condition of the fish (poor condition in chinook salmon) was a major contributor to the poor performance of salmon WM stored under hyperbaric conditions. This issue will be further discussed in a later section. It would be of interest to repeat the storage trial with salmon that are in good condition to determine, more accurately, what effect hyperbaric PM storage has on the WM from this species.

8.4 The progression toward anaerobic generation of ATP

Even though the WM was initially able to generate ATP aerobically when it was stored under hyperbaric conditions, the WM eventually became acidified and had to rely on anaerobic generation of ATP. There were three likely scenarios as to why this occurred: i) the treatment was inefficient in delivering enough oxygen to the tissue, ii) once the PCr stores had been depleted aerobic generation of ATP could not satisfy demand (PCr was "topping up" ATP levels), and iii) accumulation of waste products from aerobic generation of ATP, such as H^+ and CO_2, may have damaged the mitochondria and/or inhibited their function in some way.

ATP levels were maintained in hyperbaric WM over the first 27 h of PM storage, with bubbles forming in the WM upon depressurisation after this time. This suggested that oxygen delivery was efficient enough for the demands of oxidative phosphorylation. A rise in creatine levels (indicative of a decrease in PCr) in the hyperbaric WM coincided with depletion of ATP. This implied that ATP levels were being maintained in the WM by aerobic generation of ATP alone, rather than being "topped up" by PCr. Stopping gas flow through the hyperbaric treatment chambers (Chapter 3) essentially counteracted the delay in acidification possible with hyperbaric hyperoxia and gas flushing. This suggested that elimination of waste products was an issue for the continuation of aerobic generation of ATP lending weight to inhibition of mitochondrial respiration by CO_2 and other waste products of aerobic respiration. As discussed in Chapter 4, the (assumed) high levels of oxygen in the WM stored under hyperbaric conditions may have also poisoned the mitochondria giving the WM only one option for ATP generation: anaerobic glycolysis.

8.5 Can mitochondrial respiration be inhibited by physiological factors in vitro?

The focus of the investigation was then turned to the site of aerobic ATP generation, the mitochondria, and possible factors that may inhibit the functioning of the mitochondria (Chapter 6). As the WM inevitably became acidified during PM storage it was thought that the pH of the WM might have played a major role in inhibiting the mitochondria. Similarly, during aerobic metabolism one of the end products would have been CO_2. It was thought that even though CO_2 is a highly soluble gas with a high diffusion constant and may have been relatively quickly eliminated from the WM by diffusion when it was produced, high local concentrations may have occurred in the cell, inhibiting the mitochondria. Mitochondria were extracted from the WM and their respiration was followed in incubation mediums of various pH and CO_2 concentrations similar to physiological levels. When incubated in medium equilibrated with CO_2 concentrations of up to 10% by volume in air, it was found there was a slowing in the maximal respiration rate but the mitochondria were not inhibited as such. But when the CO_2 was increased to 15% with a corresponding pH of ~6.5, the decrease in RCR was greater than the decrease produced by low pH alone. Although the in vitro conditions were

unrealistic (very high O_2, CO_2 and substrate) the result suggested that the combination of waste products from aerobic generation of ATP may act together to inhibit oxidative phosphorylation. The role of CO_2 in inhibiting mitochondrial function in this preparation was supported by the observation in Chapter 3 that restricting the gas flow through the storage chamber under hyperbaric, hyperoxic conditions essentially counteracted the delay in acidification achieved with hyperbaric hyperoxia and gas flushing. These results suggest that a combination of high CO_2 and low pH may act to inhibit mitochondria respiration.

The focus then turned back to the ischemic WM preparation to identify if and when the mitochondria cease to function during PM storage under normobaric and hyperbaric conditions.

8.6 The change in functionality of white muscle mitochondria during post-mortem storage

It was thought that because the mitochondria were not being "used" in normobaric WM, i.e. only anaerobic metabolism was possible, that the function of the mitochondria measured by the Respiratory Control Ratio (RCR) would steadily decline during PM storage. Conversely, it was thought that because PM storage of WM under hyperbaric conditions conferred a major benefit by delaying the onset of anaerobic glycolysis, that the mitochondria would stay functional for much longer than in normobaric stored WM, i.e. mitochondrial function during PM storage would reflect WM pH. However, this was not strictly the case. Although mitochondria extracted from normobaric WM did not stay as well coupled for as long during PM storage as mitochondria from hyperbaric WM, after 10 h storage the RCR was not different to the pre-storage value, i.e. the mitochondria had not deteriorated as first thought. It was suggested that even though the mitochondria maybe "working" during the first ~10 h of PM storage, the treatment may not have been protective of mitochondrial functionality. In comparison, the mitochondria in normobaric WM were not able to function during PM storage due to the lack of oxygen. However, this seemed to be protective for mitochondrial function. In the live animal mitochondria in the WM might not be functioning during burst exercise as ATP would need to be generated at a much higher rate than could be supplied by oxidative phosphorylation. Vasoconstriction during burst exercise would

also make the WM hypoxic. Thus, the mitochondria would be shut down, ready to be activated again when the cellular environment became favourable. In other words it would be natural for the mitochondria to not function for certain periods but be "on-hold" and ready for aerobic generation when conditions were suitable. In the hyperbaric WM it was assumed that high levels of oxygen in the WM coupled with aerobic generation of ATP may have resulted in reactive oxygen species being formed (Jamieson et al. 1986; Skulachev 1996), the consequences of which would have been mild uncoupling to reduce the oxygen concentration (Skulachev 1995, 1996). Therefore, even though PM storage of WM under hyperbaric, hyperoxic conditions conferred a significant advantage in slowing the rate of PM changes in the WM, the high levels of oxygen may also have been detrimental to the continued functioning of the mitochondria.

Overall, the results suggest that increased acidification, high levels of CO_2 and hyperoxic conditions in the WM may have lead to the inhibition of aerobic generation of ATP during PM storage under hyperbaric, hyperoxic conditions.

8.7 Possible alteration of white muscle mitochondrial function by acclimation temperature

There is extensive literature showing that during cold acclimation over the course of several weeks many species of fish undergo a series of compensatory biochemical modifications (for a review, see Johnston 1993). Levels and activities of some glycolytic enzymes increase with cold acclimation along with an increase in mitochondrial volume density in red and white muscle. This is particularly evident in winter active fish, such as salmonids (Battersby & Moyes 1998; Egginton et al. 2000; St. Pierre et al. 1998). The maintenance of aerobic flux at cold temperatures is perceived to be of paramount importance. With this emphasis on aerobic flux it was thought that mullet acclimated to winter temperatures would undergo compensatory changes with the WM gaining even more benefit when stored under hyperbaric conditions compared with summer acclimated fish. However, this was not the case. WM from winter acclimated mullet stored under hyperbaric conditions did not gain as much benefit as WM from summer acclimated mullet. The summer WM had a greater ability to generate ATP aerobically during PM storage compared with winter WM. The

WM pH measurements suggested that the hyperbaric winter WM did not respire aerobically at all during the initial stages of PM storage as the pH profile was very similar to that of the normobaric summer WM. However, measurement of ATP, lactate, etc showed that there was an aerobic period of ATP generation, but this was shorter than in the summer WM.

During winter mullet become less active than in summer and consequently do not need to consume as much food as energy demand has fallen (personal observation). It is also a period when somatic growth is at its minimum and lipid stores are increased to cope with the shortage of food. During this period carbohydrate is the dominant fuel for ATP generation in rainbow trout (Kieffer et al. 1998) and red sea bream (Woo 1990). During summer, both lipid and carbohydrate would be available for ATP generation. The level of glycogen in the WM was similar in summer and winter acclimated mullet but when the biochemicals and metabolites were measured during PM storage it was clear that the winter WM did not have as much energy generating potential as summer WM. In summer WM, the period of aerobic respiration could have been fuelled by lipid, sparing the glycogen for anaerobic glycolysis later in storage. In winter WM it was possible that the only substrate available for both aerobic and anaerobic respiration was glycogen.

In fish that are more active during winter it would be likely that mitochondria numbers would increase and enzyme activity altered to maintain aerobic flux. It would be prudent for winter acclimated fish to use the more efficient process to generate ATP, i.e. aerobic metabolism, given that winter acclimated fish are probably limited to carbohydrate as substrate (Kiessling et al. 1995; Woo 1990). However, in species that are less-active in the winter, e.g. yellow-eye mullet, these compensatory mechanisms would be pointless due to the decrease in demand for energy. This has also been suggested by others studying winter-inactive species, such as sea bass (Trigari et al. 1992). Indeed, when several attempts were made to extract mitochondria from the WM in winter acclimated mullet they were found to be poorly coupled and the yield was very low. Therefore, trying to delay PM changes in winter WM by allowing aerobic generation of ATP did not result in the same benefits as seen in summer acclimated fish.

8.8 Implication of diet on post-mortem metabolism

In Chapter 5 the physical condition of the chinook salmon used for the experiments was poor. They developed a phenomenon called G-DAS (gastric-dilation air sacculitus) that was attributed to their poor quality, high lipid diet. It was possible that salmon were also deficient in some essential fatty acids and vitamins because of these dietary problems. The results of the PM storage investigation indicated that the overall muscle metabolism (e.g. enzymes levels, substrate preferences) might have changed as a result of this abnormal diet.

It was apparent through observation that the lipid content of the muscle was not as high as it usually would be. As suggested for mullet, there may also have been a reliance on carbohydrate as a substrate for energy in winter acclimated fish though salmon may be considered a winter-active fish typically having very high muscle fat reserves. If salmon WM was low in lipid (even though fish were sampled during summer) they too may have had to rely on carbohydrate for energy production, i.e. the muscle metabolism had been altered by the dietary problem.

There has been extensive research carried out into fish nutrition, mainly concerned with optimising feed conversion ratios and also improving growth rates of fish. The literature (Chapter 7) suggests that with the change of seasons, substrate preferences change in the WM and that this is a necessary process associated with gonad development relative to somatic growth. Commercial fish feed producers such as EWOS have obviously evaluated this and now produce separate feeds for both summer and winter. It would of course be of little value to feed fish dietary components that they cannot fully utilise at certain times of the year. Although fish nutrition research is very important for commercial growers there has been very little, if any research into the effects of diet on PM metabolism. If the PM muscle metabolism can be adversely affected by diet (as seen in the salmon in Chapter 5) it is then reasonable to assume that it can also be positively affected. It would be of considerable interest to pursue this area of research.

8.9 Where to from here for tissue preservation?

Although the hyperbaric, hyperoxic treatment of the ischemic WM was successful in extending the pre-rigor period it did not go so far as to preserve the tissue in its original "rested" state. The WM still acidified, be it at a slower rate, and once anaerobic generation of ATP dominated the rate of lactate accumulation was at a similar rate as that measured in both the normobaric, hyperoxic WM. In other words hyperbaric treatment postpones PM changes by prolonging oxidative metabolism and its associated energy efficiencies, but has no beneficial effect once anaerobic metabolism dominates. In the current study the results suggested that cessation of aerobic generation of ATP was due to damage of the mitochondria (Chapter 6). It is possible that the hyperbaric, hyperoxic storage of rested, ischemic WM lead to the formation of reactive oxygen species and the subsequent damage to the mitochondria. This result is consistent with that observed in the use of hyperbaric oxygen (HBO) therapy in mammals (Elayan et al. 2000).

The use of HBO therapy in the medical world is limited to a few conditions including acute traumatic ischemic injury, and aiding in skin graft and flap healing. The success of hyperbaric oxygen (HBO) therapy in such cases appears greatest if it is applied as soon as the trauma event has occurred. If treatment is delayed and partial reperfusion of the tissue occurs further damage can result due to free radical formation (Kindwall 1993). Treatment with HBO increases the oxygen content of the ischemic tissue and allows the cells to generate ATP aerobically. Although HBO therapy has positive effects in some conditions, if treatment is extended for long periods (over 2 h) any improvement of the condition may be negligible or the condition could worsen. This is due to lipid peroxidation and reactive oxygen species formation (Gregorevic et al. 2000). The gaseous compound nitric oxide (NO) has also been implicated in HBO-induced toxicity (Elayan et al. 2000). Nitric oxides toxicity may be due to its inhibition of complex I and II in the electron transport chain. This can reduce mitochondrial respiration leading to ATP depletion and eventually disruption of the active transport systems of the cell (Elayan et al. 2000).

8.9.1 Learning from organ preservation research

Research into other methods of sustaining cellular viability has focused on the preservation of organs for transplantation. At present organs, such as the heart, can only be preserved for 4 to 6 h and still be successfully transplanted (Hegge et al. 2001). Because organs are normally well perfused with blood a large body of research has concentrated on developing perfusion media that can maintain the viability of the organ. Together with hypothermic storage some organ preservation solutions, e.g. University of Wisconsin (UW) solution, have proved successful in prolonging viability for slightly longer (~10 h for hearts; Mitchell et al. 1996, Pulis et al. 2000). These solutions aim to provide the organ with substrates for energy production as well as a way of removing waste. In this regard the perfusion solutions act in a similar way to the blood. However, due to the complex functions of the blood and circulation, long term storage (weeks to months) of organs using these methods has not been possible.

Other types of organ preservation have concentrated on attempting to protect unperfused organs against ischemic damage. This research is similar to the current study in that there is application of external factors, such as oxygen or glycolytic substrates to try to maintain cell viability for the longest period. During periods of ischemia anaerobic ATP generation cannot meet the high demands of homeothermic tissue and as a result there is a continuous decline in turnover rate (Singer et al. 1993). However, the inherently high ATP demands of homeothermic tissue causes a continuous deficit of ATP to accumulate. Hypothermic storage of tissues and organs goes some way to reducing metabolic rates and the demand for ATP to maintain homeostasis. Yet, the inability to reduce the metabolic rate without causing further damage to the isolated tissue, e.g. freezing, has proved the largest barrier to extending the period between organ harvest and transplantation into the recipient. Although hypothermic storage of tissue and organs reduces the metabolic rate, and therefore the demand for energy, cells still need ATP to maintain ionic and electrical homeostasis. .

8.9.2 Learning from nature

In nature certain animals facing extreme conditions of cold and/or hypoxia are able to decrease their metabolic rate by 88%, e.g. ground squirrels (Wang & Lee 2000). If this level of metabolic rate depression could be achieved in isolated tissue then the main

barrier to long-term tissue preservation will have been crossed. So why has this not been achieved? There is a significant difference between reducing the metabolic rate of isolated tissue by reducing the temperature and the reduction of metabolic rate in animals facing hypothermic and/or hypoxic conditions. Animals that can naturally achieve states of hypometabolism do so in a strictly regulated manner (Boutilier 2001). Hibernators can control their body temperature and avoid falling below a critical metabolic rate (Singer et al. 1993). For tissue preservation purposes hypothermia may be able to reduce the metabolic rate but there is a point at which the structural integrity of the tissue is lost (Singer et al. 1993).

Teleost WM is poorly perfused (Johnston 1981), regularly faces periods of hypoxia (burst exercise), and successfully recovers. However, it is only because the WM can generate ATP rapidly by anaerobic glycolysis that the muscle cells can continue to function. There is no strategy to reduce the ATP consumption rate in order for oxidative phosphorylation to continue because the actively contracting WM fibres demand generation of ATP at a very high rate. Similarly, when the WM has been isolated from a rested fish, and is ischemic, the only option for energy generation is through anaerobic glycolysis. In the current study, when oxygen was supplied to the WM under hyperbaric, hyperoxic conditions the tissue was able to generate ATP aerobically for a period. However, once the mitochondria were not able to function and anaerobic glycolysis dominated, the change in metabolites in the WM (lactate, ATP, etc) occurred at a similar rate as that occurring in the normobaric, hyperoxic treatment (Chapter 4). This suggested that although the hyperbaric, hyperoxic treatment aided in maintaining the homeostatic ATP level in the WM it did not do so by lowering the metabolic rate to lower than that achieved by hypothermic storage, i.e. at half the acclimated temperature.

8.9.3 Decreasing the demand for ATP

So how is the metabolic rate of tissue able to be lowered in such a way that maintains structural integrity and metabolic function? The key for a whole animal to survive periods of hypometabolism, whether it be for several hours, e.g. intertidal mussels, or several weeks, e.g. overwintering frogs, is stability in the concentration of adenine nucleotides (ATP, ADP, AMP; Boutilier et al. 1997). To maintain levels of ATP under conditions of hypoxia, energy consuming processes need to be down-regulated. One

hypothesis is that metabolic suppression at a cellular level is synonymous with ion channel suppression (Boutilier et al. 1997). ATP consumption by ion channels makes up ~40% of the standard metabolic rate of tissue (Brand et al. 2000). Cells of anoxia-tolerant animals exhibit large-scale reductions in absolute Na^+/K^+ activity during anoxia without any disruptions in electrochemical potentials, cellular ion levels or ATP concentrations (Boutilier 2001). This so-called 'channel arrest' (Hochachka 1986) actively downregulates the ATP demands of cells in energy-limited states. In contrast, anoxic mammalian cells show a non-adaptive 'channel leak' response (Boutilier 2001) which raises the demand for ATP.

Another strategy for reducing the ATP demand in cells is at the site of ATP production itself, the mitochondria. In normally respiring animal cells the mitochondrial F_1F_0-ATPase acts as the site for ATP production. But during anoxia the reversible ATP synthase can run backwards and actually consumes ATP. In anoxia-tolerant animals ATP use by the F_1F_0-ATPase is limited during anoxia by a profound inhibition of the enzyme (St-Pierre et al. 2000). This leads to the maintenance of a lower mitochondrial membrane potential to a biologically viable level that saves energy. This energy saving is thought to be critical to the preservation of the cell membrane potential (Boutilier 2001).

Although hibernating animals facing harsh environmental conditions are able to lower their ATP demand they are still able to activate anaerobic ATP supply pathways (Pasteur effect) and use their extensive glycogen stores as fuel (Storey 1993). The lower metabolic rate means that ATP supply keeps pace with ATP demand. However, in some animals that face anoxia there is no reliance on anaerobic glycolysis (no Pasteur effect) when oxidative phosphorylation is not possible. Long-term survival in the absence of the Pasteur effect is only possible if ATP demand is severely reduced in the anoxic state (Storey 1988). In the blue mussel (Mytilus edulis) there is a 10-20 fold depression of metabolic heat production (Storey 1988). This 'metabolic arrest' (Storey 1993) can limit or prevent metabolic damage and greatly extend the potential survival in the arrested state. The lack of a Pasteur effect results, in part, due to the down-regulation of glycolytic enzymes via reversible protein phosphorylation (Boutilier 2001).

Overall, there is a universal commonality to what makes hypoxia and/or hypothermia tolerant animals so successful in such harsh conditions: metabolic rate is actively decreased in order to maintain ATP at homeostatic levels. In the current study hyperbaric, hyperoxia was able to maintain ischemic WM ATP levels for a short time. But without a reduction in the metabolic rate of the tissue, i.e. a reduction in ATP demand, ATP depletion and loss of cell viability was inevitable.

8.10 Concluding remarks

If normally hypoxia/hypothermia intolerant tissue could be prompted to take on the properties of hypoxia/hypothermia tolerant tissue we would be well on the way to successful long-term tissue and organ preservation. However, little is known about the signaling pathways involved in the transition to a hypometabolic state. The complexity of the cellular, biochemical, electrochemical and molecular mechanisms responsible for active metabolic rate suppression, as well as upon returning to the normal metabolic status suggest that it may be a long time before we will be able to successfully manipulate isolated tissues and organs to survive long-periods of ischemia and still be viable upon transplantation. However, it is not only the medical world that will benefit from such research. Being able to maintain tissue, whether it be fish WM, mammalian red muscle, fruits or vegetables, in a state resembling the resting condition for long periods provides any number of processing options. Such a level of control over essentially "live" products would result in minimisation of waste and increased efficiency of production, in turn maximizing the monetary as well as the nutritional value of the product. Such an achievement would also allow cost-effective distribution of fresh food around the world.

Although the task ahead is challenging the understanding that will be gained from unraveling some of natures mysteries may also allow us to solve many other unrelated problems.

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APPENDIX 1

Biochemical Methodology

The biochemical methods for the analysis of fish WM lactate, ATP, P_i, glycogen and creatine are outlined below.

Lactate

Introduction

Lactate content of WM was measured using a portable blood lactate meter (Accusport Model 1488767, Boehringer Mannheim, Germany). The neutralised sample extract was used instead of whole blood or plasma with the meter set on plasma mode. Lactate was determined by reflectance photometry via a colourimetric lactate-oxidase mediator reaction on the test strip.

Assay Method

1. Prepare lactate meter for testing as per the instructions and set in PLASMA mode.

2. Insert a lactate strip into the meter ensuring the correct code is displayed.

3. Pipette 20 μL of tissue extract (see Chapter 1, Section 3.9 'Tissue extraction') or standard onto strip.

4. Record reading.

Standard Curve Preparation

Using L-(+) Lactic acid (30% aqueous solution: Sigma L-1875) a 12 mM stock solution was made (12 mM stock solution in 0.6 M perchloric acid neutralised with KOH - 3.34 mL Lactic acid solution in 1 L or 0.168 mL in 50 mL).

Using this standard a calibration curve was obtained (Fig. Ap1).

Final concentration Lactate (mg/mL)	0.180	0.360	0.540	0.721	0.901	1.081
Final concentration Lactate (µg/mL)	180	360	540	721	901	1081
Meter Reading	1.3	2.7	4.8	6.6	8.6	9.7

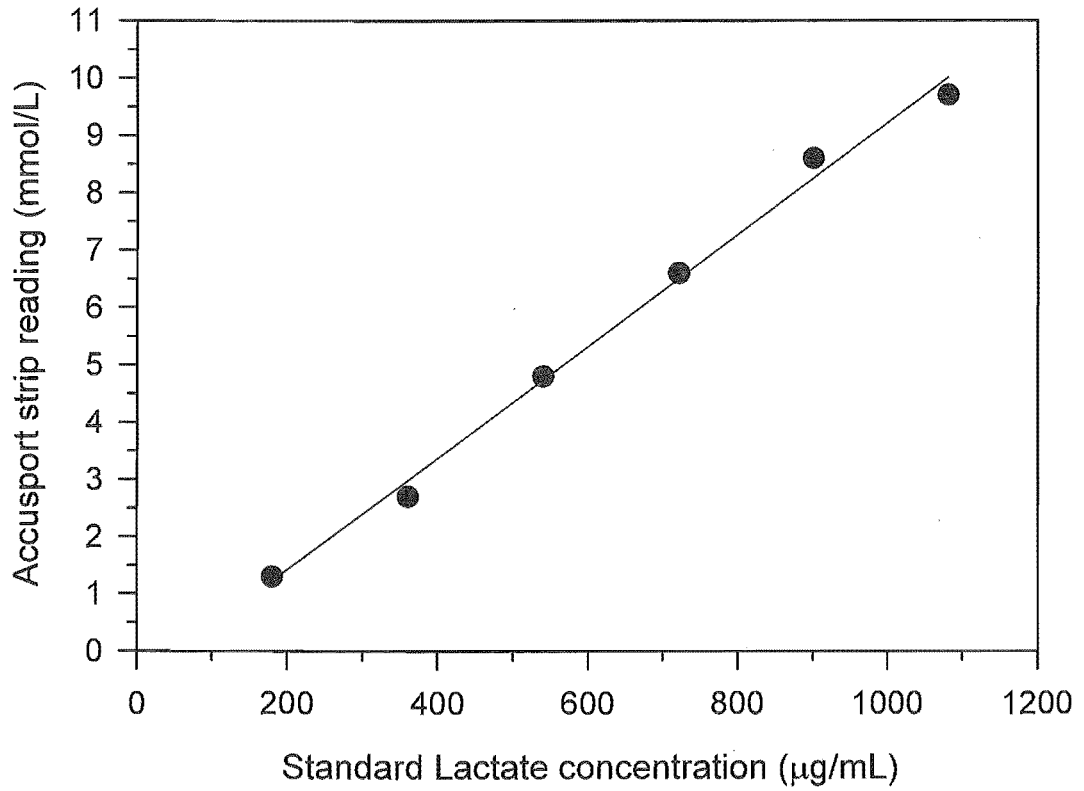

Figure Ap1: *Lactate calibration curve for Accusport measurement system. The calibration curve is described by the linear regression equation:* $y = 0.0097x - 0.530$; $r^2 = 0.99$.

Calculations

Once lactate reading have been recorded from the Accusport meter following calculation was used to determine the concentration of lactate in the WM sample.

Convert the meter reading to µg lactate/mL using $y = 0.0097x - 0.530$ (linear regression equation from standard curve, Fig. Ap1).

e.g. if reading is 0.8 mM

then μg lactate/mL $= \frac{0.8 + 0.530}{0.0097}$

$= 137.1$ μg lactate/mL

μg lactate/g tissue = 137.1/(sample wgt (g)/0.5 mL perc. acid + mL to neut.)

= 137.1/(0.1 g/0.525 mL)

= 719.85 μg lactate/g muscle mass

μmoles lactate/g tissue= 719.85 / 90.08 g/mol (M.W. lactate = 90.08)

= 7.99 μmoles lactate/g muscle mass

Adenosine triphosphate

Introduction

ATP content of WM was determined using an ultraviolet spectrophotometric analysis as for whole blood (Sigma Diagnostics 366-A). The ATP assay was not specific for ATP, it reads GTP, ITP and UTP as ATP. The ATP is assayed using phosphoglyceric phosphokinase (PGK) and glyceraldehydes phosphate dehydrogenase (GAPD) as follows:

ATP + 3-phosphoglycerate → ADP + 1,3 diphosphoglycerate

1,3 diphosphoglycerate + NADH → glyceraldehyde-3-P + NAD + P

The reduction in the amount of NADH present is stoichiometric to the amount of ATP in the sample.

Reagents

1. PGA Buffered Solution (Sigma Cat. No. 366-1)

2. NADH Solution - using NADH (disodium salt, grade II, Roche, Germany) make up a 1 mg/mL solution using 0.01 mol/L NaOH.

3. GAPD/PGK Enzyme Mix (Sigma Cat. No. 366-2)

4. ATP - the ATP used in the preparation of the standard curve is the disodium dihydrogen salt (BDH Chemical 420083H Lot No. K22702931 613 FW 605.19).

Assay Method

1. Into a microcuvette (2.5 mL, LPI 112117) add 0.333 mL PGA Buffered solution.
2. Add 0.1 mL NADH solution
3. Add 0.517 mL distilled water
4. Add 0.05 mL of fish muscle extract (see Chapter 2, Section 2.8 'Tissue extraction') or prepared standard
5. Mix.
6. Read initial absorbance at 340 nm with WATER as reference. Absorbance should be no less than 0.6 (if it is there may be a problem with the NADH solution)
7. Add 0.0133 mL GAPD/PGK Enzyme Mix
8. Mix
9. Read final absorbance at 340 nm and continue readings until the lowest absorbance is read (~5-10 mins).

Standard Curve Preparation

Use the ATP described above to make up standards.

Weigh out 9.7 mg of ATP into a 10 mL volumetric and dissolve in distilled water which will give a final concentration of 1.6 μmoles/mL (want range of standard curve 0 - 0.08 μmoles in cuvette).

Use this standard to construct the following standard curve (Fig. Ap2).

Final concentration ATP (µg/mL)	0	242	484	726	968
PGA buffer (µL)	333	333	333	333	333
NADH solution (µL)	100	100	100	100	100
Distilled water (µL)	567	554.5	542	529.5	517
ATP std (µL)	0	12.5	25	37.5	50

After initial reading:

GAPD/PGK Enzyme Mix (µL)	13.3	13.3	13.3	13.3	13.3

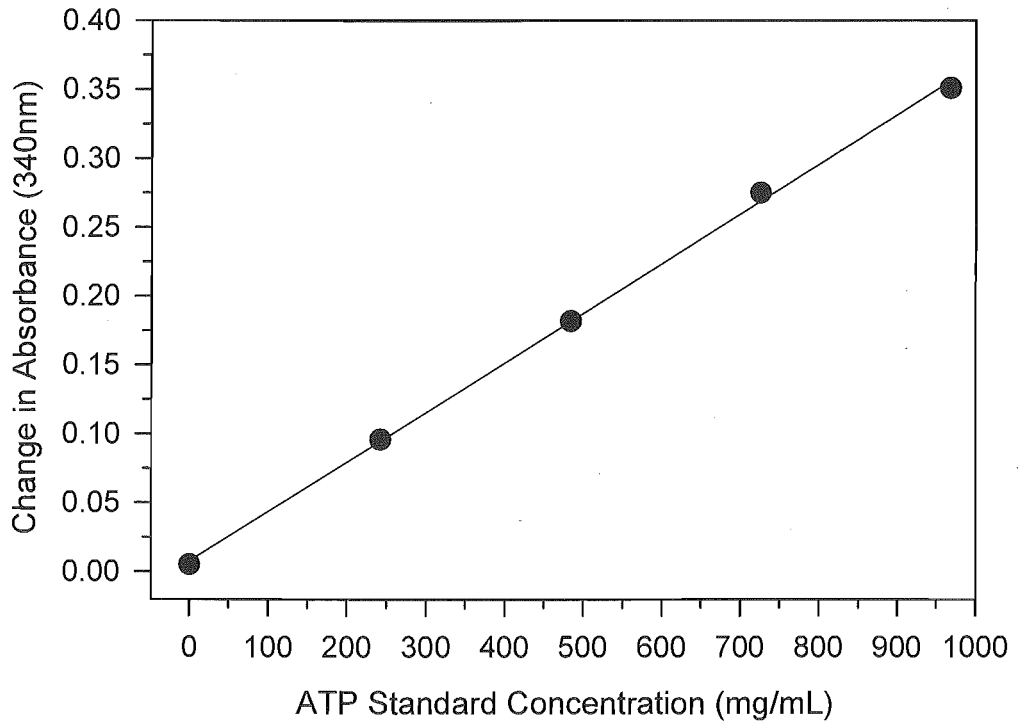

Figure Ap2: *ATP calibration curve. The calibration curve is described by the linear regression equation:* $y = 3.599 \times 10^{-4} x + 0.0076$; $r^2 = 0.99$.

Calculations

Once you have your two absorbance readings use the following calculation to work out your concentration of ATP.

Convert change in absorbance to µg ATP/mL extract using $y = 3.599 \times 10^{-4}x + 0.0076$ (linear regression equation from standard curve).

e.g. if change in absorbance = 0.299 (i.e. 0.831 - 0.532)

$$\mu g \text{ ATP/mL extract} = \frac{0.299 - 0.0076}{3.599 \times 10^{-4}}$$

= 809.67 µg ATP/mL extract

µg ATP/g tissue = 809.67/(sample wgt (g)/0.5 mL perc. acid + mL to neut.)

= 809.67/(0.12 g/0.528 mL)

= 3562.5 µg ATP/g muscle mass

µmoles ATP/g tissue = 3562.5 / 605.19 g/mol (M.W ATP = 605.19)

= 5.89 µmoles ATP/g muscle mass

Inorganic phosphate

Introduction

The P_i concentration of fish WM was determined using a method based on a colourimetric assay described by Sigma Diagnostics (Cat. No. 670-A). The phosphate ions react with ammonium molybdate at acid pH, forming ammonium phosphomolybdate. Reaction of this compound produces a blue phosphomolybdenum complex:

$$\text{Phosphorus} + \text{Ammonium Molybdate} \xrightarrow{\text{Acid pH}} \text{Ammonium Phosphomolybdate}$$

$$\text{Ammonium Phosphomolybdate} + \text{Aminoaphyholsulfonic acid} \rightarrow \text{Heteropolymolybdenum (Blue)}$$

Reagents

1. Acid molybdate solution - In a 200 mL volumetric flask weigh 2.5 g Ammonium Molybdate (Lot 55933; May & Baker Ltd, England; M.W. 1235.86). Make up to the mark with 1.25 mol/L sulphuric acid.

2. Potassium dihydrogen orthophosphate (KH_2PO_4) - The KH_2PO_4 used in the preparation of the standard curve is Analytical Grade (AnalaR 10203 4B, BDH).

Assay Method

1. Into a microcuvette (2.5 mL, LPI 112117) add 0.9 mL distilled water

2. Add 0.1 mL Acid Molybdate Solution.

3. Add 0.01 mL of fish muscle extract (see Chapter 1, Section 3.9 'Tissue extraction') or prepared standard.

4. Mix.

5. Read absorbance immediately at 340 nm against blank (0.91 mL water + 0.1 mL Acid Molybdate solution).

Standard Curve Preparation

Use KH_2PO_4 as the source of P_i (KH_2PO_4 MW = 136.09; P_i = 30.97 i.e. P_i makes up 22.8% of KH_2PO_4). Need 337 µg of P_i in 1 mL (0.337 g P_i in 1 L) i.e. 0.337/22.8% = 1.48 g of KH_2PO_4 in 1 L to get 337 µg of P_i in 1 mL. Therefore make up 147.8 mg of KH_2PO_4 in 100 mL.

Using this standard the following table can be used to construct a standard curve (Fig. Ap3).

Final conc P_i (µg/mL)	0	111	133	156	178	200	222	333
Dist. water (µL)	3000	2990	2988	2986	2984	2982	2980	2970
Acid molybdate (µL)	300	300	300	300	300	300	300	300
P_i standard (µL)	0	10	12	14	16	18	20	30

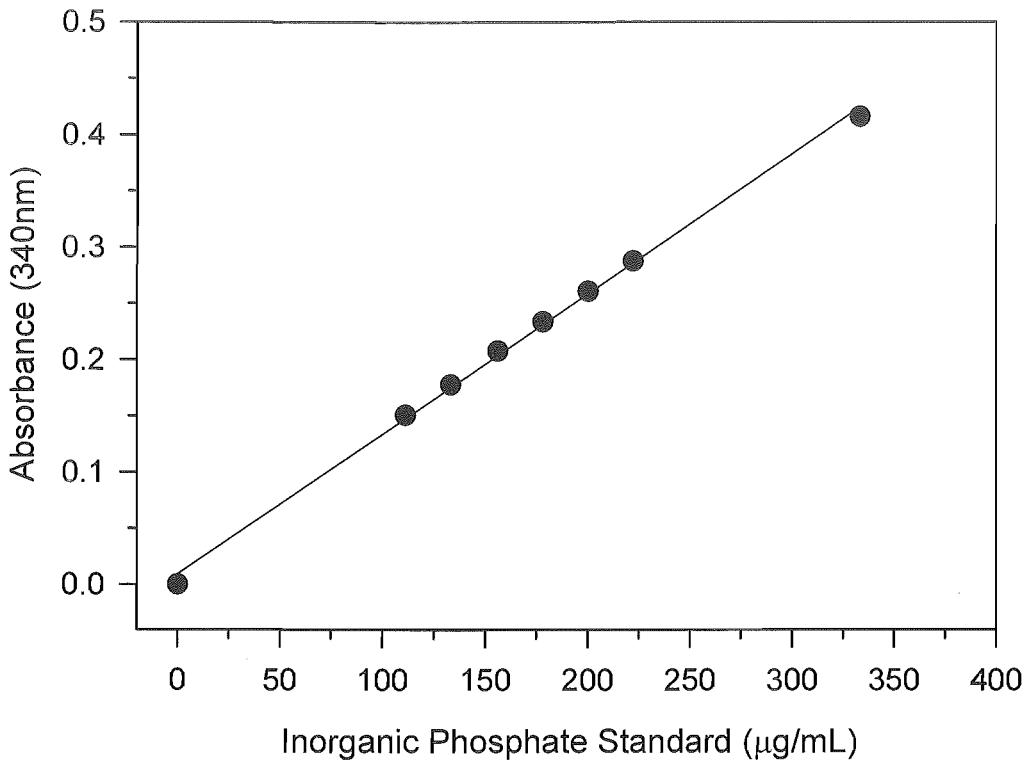

Figure Ap3: *Inorganic phosphate calibration curve. The calibration curve is described by the linear regression equation:* $y = 1.247 \times 10^{-3} x + 0.009$; $r^2 = 0.99$.

Calculations

Once you have your absorbance use the following calculation to work out in µg/mL

Convert absorbance to µg/mL using $y = 1.247 \times 10^{-3}x + 0.009$

e.g. absorbance = 0.240

µg P_i /mL extract $= \dfrac{0.240 - 0.009}{1.247 \times 10^{-3}}$

= 185.24 µg P_i /mL extract

µg P_i /g tissue = 185.24/(sample wgt (g)/0.5 mL perc. acid + mL to neut.)

= 185.24/(0.10 g/0.530 mL)

= 981.80 µg P_i /g muscle mass

µmoles P_i /g tissue = 981.80 / 30.97 g/mol (M.W P_i = 30.97)

= 31.70 μmoles P_i/g muscle mass

Creatine

Introduction

The total creatine concentration of fish WM was determined using a method based on that described by Eggleton et al. (1943) that was modified so the assay could be performed in a 3 mL cuvette. The assay used is based on the chemical reaction:

creatine + α – naphthol → coloured complex (pink)

Reagents

1. Alkali Stock Solution – 30 g NaOH + 80 g Na_2CO_3 make up to 500 mL with dist. water.

2. Diacetyl Stock Solution - 1% made up with dist. water. Make up 1:20 dilution prior to use.

3. α-Naphthol - 1% stock solution in alkali. Make as needed as only keeps 2-3 h.

4. Creatine (Sigma Cat. No. C3630).

Assay Method

1. Into a micro-cuvette (2.5 mL, LPI 112117) add 0.2 mL distilled water and 0.1 mL tissue extract (see Chapter 1, Section 3.9 'Tissue extraction') or prepared standard sample.

2. Add 0.2 mL α-naphthol in alkali and 0.1 mL diacetyl diluted solution.

3. Add 0.4 mL dist. water to make up to 1 mL.

4. Mix and allow to stand for 30 min for colour development.

5. Dilute assays by 10 (0.25 mL sample to 2.25 mL water)*

6. Read absorbance at 525 nm

* The point at which the dilution was made was found to be wrong. The samples should have been diluted at the start of the assay, not the end. This resulted in readings over ~20 μmol/g being inaccurate (under-estimated).

Standard Curve Preparation

Creatine standard solution – 60 mg of creatine (Sigma Cat. No. C3630) was weighed into a 100 mL volumetric flask and made up to the mark with distilled water.

Using this standard the following table can be used to construct a standard curve (Fig. Ap4).

Final conc creatine (μg/mL)	0	10	20	30	40	50	60
Dist. water (μL)	700	683	667	650	633	617	600
α-naphthol (μL	200	200	200	200	200	200	200
Diacetyl (μL)	100	100	100	100	100	100	100
Creatine standard (μL)	0	17	33	50	67	83	100

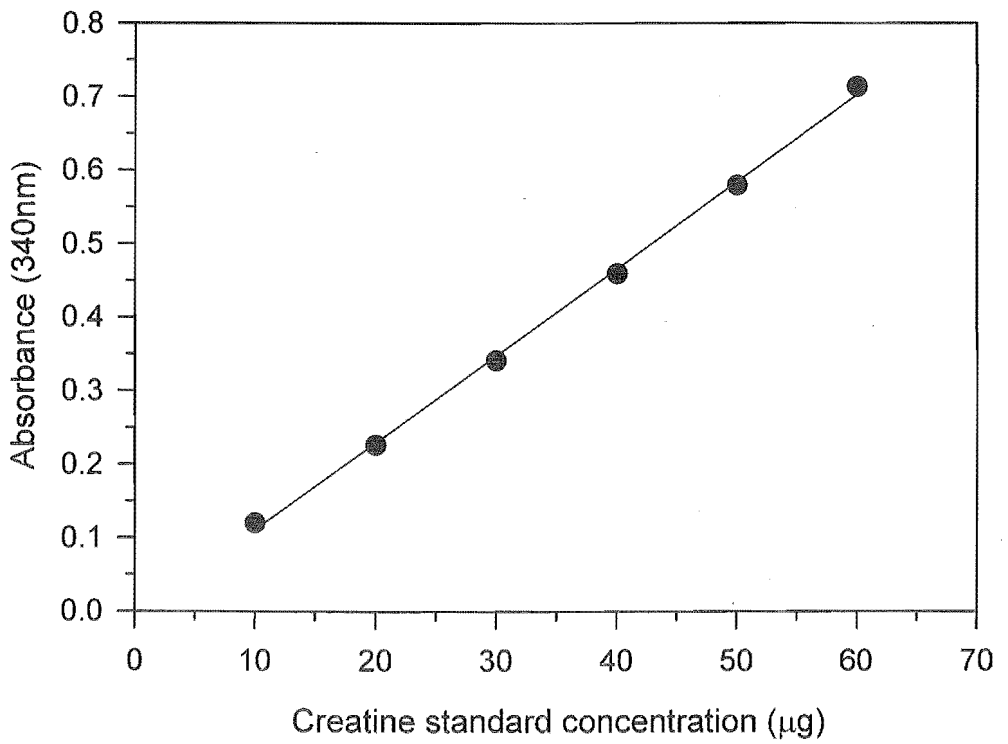

Figure Ap4: *Creatine calibration curve. The calibration curve is described by the linear regression equation:* $y = 0.01183\,x - 0.00787$; $r^2 = 0.99$.

Calculations

Once you have your absorbance use the following calculation to convert the absorbance to μg creatine/mL extract using $y = 0.01183x - 0.00787$ (linear regression equation from standard curve).

e.g. if absorbance is = 0.5

$$\mu g \text{ creatine/mL extract} = \frac{0.5 + 0.00787}{0.01183}$$

= 42.93 μg creatine/mL extract

μg creatine/g tissue = 42.93/(0.1 x sample wgt (g)/0.5 mL perc. acid + mL to neut.)

= 42.93/(0.012 g/0.528 mL)

= 1888.92 μg creatine/g muscle mass

μmol creatine/g = 1888.92 / 131.1 g/mol (M.W creatine = 131.1)

= 14.41 μmoles creatine/g muscle mass

Reference

Eggleton, P.; Elsden, S.R.; Gough, N. 1943. The estimation of creatine and of diacetyl. *Biochemical Journal 37*: 526-529.

Glycogen

Introduction

This method is used to determine the concentration of glycogen in fish WM. The assay is based on the method described by Keppler and Decker (1974) which has been modified in order for the assay to be carried using a Reflotron (Roche Diagnostics) machine.

Sample pipetted onto glucose strip:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \delta\text{-D} - \text{gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{indicator} \xrightarrow{\text{POD}} \text{dye} + H_2O$$

Glycogen in the fish WM is broken down to glucose which is then measured on the Reflotron glucose strip.

Reagents

1. Potassium hydrogen carbonate ($KHCO_3$ 1 mol/L) - Weigh 2 g and make up to 20 mL with distilled water.

2. Acetate Buffer - 4.8 mL glacial acetic acid + 9.75 g sodium acetate and make up to 1 L)

3. Amyloglucosidase/Glucose Solution - weigh 20 mg of amyloglucosidase (Roche: kept in fridge) and 2.34 mg of glucose (BDH) into a 10 mL volumetric flask and make up to the mark with acetate buffer. The added glucose acts as an internal standard during the assay.

4. Glucose strips - Roche product (Cat. No. 744948: 2 X 15 tests)

5. Glucose - the glucose used in the preparation of the standard curve is the BDH product

Assay Method

1. Take 0.1 mL of homogenised sample (immediately after ultra-turraxing DO NOT use centrifuged sample) and add 0.05 mL $KHCO_3$ and 0.5 mL of amyloglucosidase/glucose solution.

2. Measure glucose on Reflotron test strip.

3. Incubate rest of sample at 40 °C (water bath) on wheel for 2.5 h.

4. Stop incubation by removing tubes from bath and placing on ice.

5. Measure glucose again on the Reflotron.

N.B. Remember to shake samples before testing.

Standard Curve Preparation

As the glycogen in fish WM is broken down in the assay to form glucose, the glucose described above was used to make up standards.

Weigh out 29.28 mg of glucose into a 10 mL volumetric and dissolve in distilled water which will give a final concentration of 2.5 μmoles/mL.

Use this standard to construct the following standard curve (Fig. Ap5):

Final concentration Glycogen (μg/mL)	**90**	**180**	**270**	**360**	**450**
Amyloglucosidase solution (μL)	500	500	500	500	500
$KHCO_3$ (μL)	50	50	50	50	50
Glucose std (μL)	20	40	60	80	100
Water (μL)	80	60	40	20	0

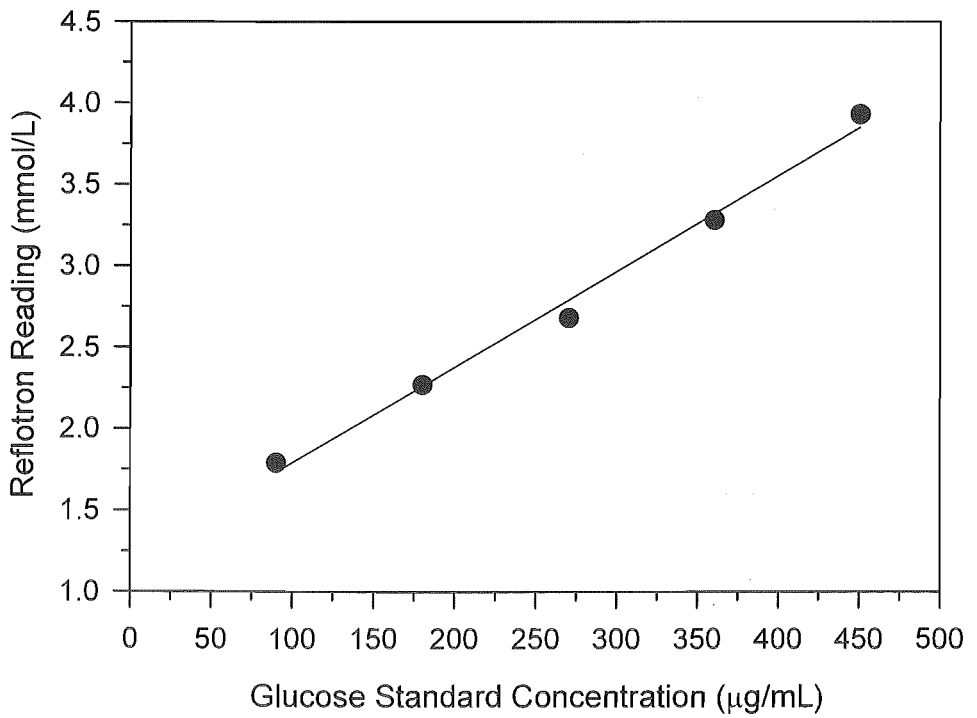

Figure Ap5: *Glycogen calibration curve. The calibration curve is described by the linear regression equation:* $y = 0.0059\,x + 1.023$; $r^2 = 0.99$.

Calculations

Once you have your two readings from the Reflotron use the following calculation to work out your concentration of glycogen:

Convert change in mmol/L to μg glucose/mL extract using $y = 0.0059x + 1.203$

e.g. if change in reading = 1.416 mM

$$\mu g \text{ glucose/mL assay} = \frac{1.416 - 1.203}{0.0059}$$

$$= 36.10 \ \mu g \text{ glucose/mL assay}$$

$$\mu g \text{ glucose/g tissue} = \frac{36.10 \ \mu g \text{ glucose/mL assay}}{((0.1 \text{ mL x sample wgt (g)}/0.5 \text{ ml perc. acid + mL to neut.}))/ 0.65)}$$

$$= 36.10/((0.1 \text{ mL x } 0.10 \text{ g}/0.525 \text{ mL})/0.65)$$

$$= 1231.91 \ \mu g \text{ glucose/g muscle mass}$$

Glycogen content of the tissue is described as μmol of glucosyl units per gram tissue.

There are 162 grams of glycosyl units per mole of glucose

$$= \frac{1231.91 \ \mu g \text{ glucose/g muscle mass}}{162}$$

$$= 7.60 \ \mu mol \text{ glucosyl units/g muscle mass}$$

Reference

Keppler, D.; Decker, K. 1974. Glycogen: determination with amyloglucosidase. *In*: Bergmeyer, H.V. *ed.* Methods of Enzymatic Analysis. Second edition. Academic Press, New York. Pp 1127-1131.

APPENDIX 2

Calculation of lactate dissociation to hydrogen ions

Because lactic acid is a weak acid, when it dissociates in solution the [H^+] is much less than the original lactic acid concentration. At equilibrium the following changes occur in the 0.07 mol/L lactate solution (remembering that the lactate concentration in the WM can reach ~70 μmol/g muscle mass):

	[HA]	[H_3O^+]	[A^-]
Initial concentration	0.07 *M*	0	0
Change to reach equilibrium	-*x*	+*x*	+*x*
Concentration at equilibrium	0.07 *M* - *x*	*x*	*x*

In terms of *x*, then at equilibrium,

$$K_a = \frac{[A^-][H_3O^+]}{[HA]}$$

$$= \frac{x^2}{0.07\ M - x}.$$

When *x*, the hydrogen ion concentration, is very small compared with 0.07 mol/L (less than about 5% of this value), this equation may be simplified to:

$$K_a = \frac{x^2}{0.07\ M}.$$

This rearranges to

$$x = 0.07\ M \times K_a$$

Since $x = [H_3O^+]$ and $K_a = 1.62 \times 10^{-4}\ M$ (Merck Index):

$$[H_3O^+] = 0.07\ M \times (1.62 \times 10^{-4}\ M)$$

$$= 3.37 \times 10^{-3}\ M$$

The approximation $x << 0.07\ M$ is valid as $1.62 \times 10^{-4}\ M << 0.07\ M$

Reference

Atkins, P.W. 1989. General chemistry. Scientific American Books, New York. Pp 534.